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The Role of Matrix Metalloproteinases in Placental Remodeling and Vascular Reactivity in Complicated Pregnancies.

by

Shaila Jamaluddin Merchant

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Master of Science*

Department of *Physiology*

Edmonton, Alberta
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Sincerely,

Ian P. Crocker — Date: June 5, 2003

Philip N. Baker — Date: June 5, 2003

Dympna Tansinda — Date: June 6 2003

Sandra T. Davidge — Date: June 11, 2003

Larry J. Guilbert — Date: June 11, 2003

We the undersigned hereby grant permission to **Shaila J. Merchant** to include our accepted publication "The effects of preeclampsia and oxygen environment on endothelial release of matrix metalloproteinase-2" in her thesis, entitled "The Role of Matrix Metalloproteinases in Placental Remodeling and Vascular Reactivity in Complicated Pregnancies." This article has been accepted for publication in "Hypertension in Pregnancy" and is in press.

Sincerely,

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ABSTRACT

Matrix metalloproteinases (MMPs) are a diverse family of enzymes that mediate placental remodeling and vascular reactivity. Changes in the levels of MMPs and their inhibitors (TIMPs) in pregnancy may contribute to the pathophysiology of preeclampsia and intrauterine growth restriction. Placental explants from IUGR pregnancies were found to secrete significantly less MMP-2 and TIMP-1 compared to explants from normal pregnancies, suggesting that impaired placental remodeling may be due, in part, to altered levels of MMPs and TIMPs. Fetal-derived human umbilical vein endothelial cells (HUVECs) from preeclamptic pregnancies were found to secrete significantly more MMP-2, TIMP-1 and TIMP-2 compared to HUVECs from normal pregnancies, and conditions of low oxygen did not stimulate normal cells to mimic this behaviour. Plasma from women with preeclampsia significantly enhanced myogenic tone and impaired endothelium-dependent vasodilation in isolated arteries, and contrary to our hypothesis, MMP inhibition failed to reverse these effects. These findings suggest that MMPs play an important role in pregnancy complications.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND UNITS

CHAPTER 1. RATIONALE, HYPOTHESES AND LITERATURE REVIEW. 1

1.1 RATIONALE 1

1.2 HYPOTHESES 2

1.3 EARLY DEVELOPMENT OF THE HUMAN PLACENTA AND ESTABLISHMENT OF THE PLACENTAL CIRCULATION 4

1.4 THE PLACENTA IN COMPLICATED PREGNANCIES 7

1.5 MATRIX METALLOPROTEINASES 8

1.5.1 Overview 8

1.5.2 The importance of MMPs in ongoing placental development 10

1.6 EFFECTS OF OXYGEN ON PLACENTAL DEVELOPMENT 11

1.6.1 Oxygen levels in early gestation 11

1.6.2 Models of placental hypoxia 12

1.6.3 Mechanisms of Oxygen Sensing 13

1.6.4 Oxygen regulation of MMPs and TIMPs 15

1.7 MATERNAL VASCULAR PATHOPHYSIOLOGY OF PREECLAMPSIA 16

1.8 ENDOTHELIAL DYSFUNCTION IN PREECLAMPSIA 18

1.8.1 The endothelium in normal physiology 18

1.8.2 Maternal endothelial activation in preeclampsia 19

1.8.3 Evidence of endothelial activation in the fetoplacental compartment of pregnancies complicated by preeclampsia 20

1.9 EVIDENCE OF MATERNAL VASCULAR DYSFUNCTION IN PREECLAMPSIA 21

1.9.1 Resistance vessels and smooth muscle in normal physiology 21

1.9.2 Local control of blood flow – the vascular myogenic response 22

1.9.3 Signal transduction mechanisms underlying the myogenic response 23

1.9.4 The role of the endothelium in the myogenic response 24

1.9.5 Myogenic tone in pregnancy 25

1.9.6 Myogenic tone in preeclampsia 25

1.10 CIRCULATING FACTORS IN PREECLAMPSIA 26

1.10.1 In vitro studies 26

1.10.2 Identity of the circulating factor 28

1.10.3 Effects of circulating factors on endothelial release of vasoconstrictors and vasodilators 29

1.10.4 The role of MMPs in vascular function 30
1.11 SUMMARY 31
1.12 REFERENCES 33

CHAPTER 2. MATRIX METALLOPROTEINASE RELEASE FROM PLACENTAL EXPLANTS FROM PREGNANCIES COMPLICATED BY INTRAUTERINE GROWTH RESTRICTION. 52
2.1 INTRODUCTION 52
2.2 MATERIALS AND METHODS 54
2.2.1 Tissue Collection and Experimental Protocol 54
2.2.2 Gelatin Zymography 56
2.2.3 Western Blot Analysis 57
2.2.4 Statistics 58
2.3 RESULTS 59
2.3.1 Comparison between normal and IUGR explants for release of MMP and TIMP at different oxygen levels 59
2.3.2 The effect of oxygen level on MMP and TIMP release from normal and IUGR explants 60
2.4 DISCUSSION 61
2.5 ACKNOWLEDGEMENTS 63
2.6 REFERENCES 64

CHAPTER 3. THE EFFECTS OF PREECLAMPSIA AND OXYGEN ENVIRONMENT ON ENDOTHELIAL RELEASE OF MATRIX METALLOPROTEINASE-2. 73
3.1 INTRODUCTION 73
3.2 MATERIALS AND METHODS 74
3.2.1 HUVEC Isolation and Culture 74
3.2.2 Preparation of HUVEC-Conditioned Medium 75
3.2.3 Gelatin Zymography 76
3.2.4 Western Blot Analysis 77
3.2.5 LDH Assays for Cell Lysis 78
3.2.6 Statistics 79
3.3 RESULTS 79
3.4 DISCUSSION 81
3.5 ACKNOWLEDGEMENTS 85
3.6 REFERENCES 86

CHAPTER 4. THE ROLE OF MATRIX METALLOPROTEINASES IN VASCULAR REACTIVITY: IMPLICATIONS FOR NORMAL PREGNANCY AND PREECLAMPSIA. 96
4.1 INTRODUCTION 96
4.2 MATERIALS AND METHODS 98
4.2.1 Animals and tissue collection 98
4.2.2 Plasma collection protocol 100
4.2.3 Experimental protocols 101

4.2.4 Calculations.....102
4.2.5 Data analysis.....102
4.3 RESULTS.....103
4.3.1 The effects of GM6001 on myogenic tone and endothelium-dependent relaxation.....103
4.3.2 The effects of plasma from non-pregnant, normal pregnant, and preeclamptic women on myogenic tone and endothelium-dependent relaxation.....104
4.3.3 The effects of GM6001 on myogenic tone and endothelium-dependent relaxation in vessels incubated in plasma from non-pregnant, normal pregnant and preeclamptic women.....104
4.4 DISCUSSION.....105
4.5 REFERENCES.....108

CHAPTER 5. GENERAL DISCUSSION......119
5.1 SUMMARY.....119
5.2 ALTERED LEVELS OF MMPS AND THEIR INHIBITORS IN PLACENTAL EXPLANTS FROM PREGNANCIES COMPLICATED BY IUGR.....120
5.3 THE PLACENTAL EXPLANT MODEL.....121
5.4 ENDOTHELIAL ACTIVATION IN CELLS FROM THE FETOPLACENTAL COMPARTMENT OF PREECLAMPTIC PREGNANCIES.....122
5.5 OXYGEN-MEDIATED MMP/TIMP RELEASE IN PLACENTAL EXPLANTS AND HUVECs: CONFLICTING RESULTS?.....123
5.6 MMPS REDUCE MYOGENIC TONE AND ENHANCE ENDOTHELIUM-DEPENDENT VASODILATION IN THE MESENTERIC VASCULATURE124
5.7 SIGNIFICANCE OF EXPERIMENTS.....126
5.8 REFERENCES.....128
APPENDIX A: OPTIMIZATION OF EXPERIMENTAL PROTOCOLS.....132
APPENDIX B: VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) EXPERIMENTS.....138

LIST OF TABLES

Table 2.1 Patient demographics of women at St. Mary's Hospital, Manchester, United Kingdom, from whom placentas were obtained. 68

Table 3.1 Patient demographics of women from Royal Alexandra Hospital, Edmonton, Alberta, from whom umbilical cords were obtained. 91

Table 4.1 Patient demographics of women from Royal Alexandra Hospital, Edmonton, Alberta, from whom plasma samples were obtained. 112

Table B.1 Sequence of oligonucleotide primers using for RT-PCR analysis of Flt-1 and KDR/Flk-1. 149

LIST OF FIGURES

Figure 1.1 The structure of the human placenta.....48

Figure 1.2 Activation of pro-MMP-2 on the surface of endothelial cells.....49

Figure 1.3 Vascular changes during normal pregnancy and preeclampsia.....50

Figure 1.4 An example of myogenic tone in small arteries and arterioles.....51

Figure 2.1 Comparison between explants from normal and IUGR pregnancies for release of MMP-2 and MMP-9 at different oxygen levels. 69

Figure 2.2 Comparison between explants from normal and IUGR pregnancies for release of TIMP-1 at different oxygen levels. 70

Figure 2.3 The effect of oxygen level on MMP-2 and MMP-9 release from explants from normal and IUGR pregnancies. 71

Figure 2.4 The effect of oxygen level on MMP-2 and MMP-9 release from explants from normal and IUGR pregnancies. 72

Figure 3.1 Gelatin zymography of MMP-2 release from HUVECs from normal pregnant and preeclamptic women.....92

Figure 3.2 Western blot analysis of TIMP-1 and TIMP-2 release from normal pregnant and preeclamptic HUVECs. 93

Figure 3.3 Gelatin zymography of MMP-2 release from HUVECs from normal pregnancies under varying oxygen conditions. 94

Figure 3.4 TIMP-1 and TIMP-2 release from HUVECs from normal pregnancies under varying oxygen conditions. 95

Figure 4.1 The effect of GM6001 on myogenic tone and endothelium-dependent relaxation in mesenteric arteries.....113

Figure 4.2 The effect of 2% pooled plasma from non-pregnant, normal pregnant and preeclamptic pregnancies on myogenic tone in mesenteric arteries (1 hour pre-incubation).....114

Figure 4.3 The effect of 2% pooled plasma from non-pregnant, normal pregnant and preeclamptic pregnancies on endothelium-dependent relaxation to methacholine in mesenteric arteries (1 hour pre-incubation). 115

Figure 4.4 The effect of GM6001 on myogenic tone in vessels co-incubated in the plasma of non-pregnant, normal pregnant and preeclamptic women (1 hour pre-incubation). 116

Figure 4.5 The effect of GM6001 on endothelium-dependent relaxation in vessels co-incubated in the plasma of non-pregnant, normal pregnant and preeclamptic women (1 hour pre-incubation). 117

Figure 4.6 MMP interactions in the vasculature. 118

Figure 5.1 The role of MMPs in the pathophysiology of preeclampsia and/or IUGR. 131

Figure A.1 Fluorescence immunostaining of HUVECs with endothelial-specific von Willebrand factor (vWF). 144

Figure A.2 Evaluation of the sensitivity of the zymography assay. 145

Figure A.3 Levels of endogenous metalloproteinases in serum-containing M199 medium 146

Figure A.4 MMP-2 accumulation in medium over time 147

Figure A.5 Western blot analysis of conditioned medium from HT1080 cells as positive controls for MMP-2/9 and TIMP-1/2. 148

Figure B.1 The effect of VEGF on metabolic capacity in HUVECs from normal pregnancies. 150

Figure B.2 The effect of VEGF on MMP-2 release from HUVECs. 151

Figure B.3 The effect of endogenous and exogenous VEGF inhibition on MMP-2 release from HUVECs. 152

Figure B.4 RT-PCR analysis of Flt-1 and KDR/Flk-1 in HUVECs. 153

ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis.

°C ..... degrees Celsius

ANOVA ..... analysis of variance

AP-1 ..... activating protein-1

AT_1 ..... angiotensin 1 receptor

ATP ..... adenosine triphosphate

B-B ..... Berger-Broida units

bp ..... base pair

BP ..... blood pressure

Ca^{2+} ..... calcium ion

$CaCl_2$ ..... calcium chloride

cAMP ..... cyclic adenosine monophosphate

cGMP ..... cyclic guanidine monophosphate

CGRP ..... calcitonin gene-related peptide

CO_2 ..... carbon dioxide

CT ..... cytotrophoblast

DMEM .... Dulbecco's modified eagles medium

EC_{50} .... effective dose eliciting 50 % response

ECGS..... ..... endothelial cell growth supplement

ECM .... extracellular matrix

EDTA .... ethylenediaminetetraacetic acid di-sodium salt

et al...... ..... *et alii* (Latin, 'and others')

EtBr .... ethidium bromide

EtOH..... ..... ethanol

ET_A .... endothelin type A receptor (on vascular smooth muscle)

ET_B .... endothelin type B receptor (on endothelium)

FBS..... ..... fetal bovine serum

Flk-1 .... fetal liver kinase (VEGF receptor-2)

Flt-1 ....*fms*-like tyrosine kinase (VEGF receptor-1)

g..... ..... acceleration due to Earth's gravity (9.8 $m \cdot s^{-2}$)

g..... ..... gram(s)

Gly..... ..... glycine (amino acid)

h..... ..... hour(s)

H_2O..... ..... water

HIF-1 .... hypoxia inducible factor-1

HCl .... hydrochloric acid

HUVECs..... ..... human umbilical vein endothelial cells

i.e. .... *id est* (Latin, 'that is')

IgG .... Non-specific mouse immunoglobulin G

IκB .... inhibitory subunit for NFκB

IUGR .... intrauterine growth restriction

IHC .... immunohistochemistry

KCl .... potassium chloride

kD .... kilodalton

l .... litre(s)

LDH .... lactate dehydrogenase

Leu .... leucine (amino acid)

m .... meter(s)

M .... moles·l^{-1}

M199 .... medium for endothelial cells

$MgCl_2$ .... magnesium chloride

min .... minute(s)

MLC .... myosin light chain

MLCK .... myosin light chain kinase

MLCP .... myosin light chain phosphatase

MMP .... matrix metalloproteinase

mRNA .... messenger ribonucleic acid

MT-MMP .... membrane type matrix metalloproteinase

MTT .... 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide

n..... ..... number of animals

N_2..... ..... nitrogen

N/A..... ..... not applicable

NaCl .... sodium chloride

NAD(P)H .... coenzyme nicotinamide adenine dinucleotide (phosphate)

NaN_3 .... sodium azide

N/D..... ..... not determined

NFκB..... ..... nuclear factor kappa B

N-terminal..... ..... amino-terminal

O_2..... ..... molecular oxygen

OD..... ..... optical density

p value .... probability (of incorrectly rejecting the null hypothesis)

PBS..... ..... phosphate buffered saline

PCR .... polymerase chain reaction

pH..... ..... logarithmic unit measuring acidity

PlGF .... placenta growth factor

RNA .... ribonucleic acid

RT..... ..... reverse transcription

s .... second(s)

SD..... ..... standard deviation of the mean

SDS .... sodium dodecyl sulphate

SEM..... ..... standard error of the mean

ST .... syncytiotrophoblast

TIMP .... tissue inhibitor of metalloproteinases

TPBS .... phosphate buffered saline with Tween

Tris-Cl .... tris[hydroxymethyl]-amino methane hydrochloride

VEGF .... vascular endothelial growth factor

VEGFR..... ..... VEGF receptor

vWF..... ..... von Willebrand factor

Mathematical prefixes

k..... ..... kilo (10^{3})

c .... centi (10^{-1})

m..... ..... milli (10^{-3})

μ..... ..... micro (10^{-6})

n..... ..... nano (10^{-9})

CHAPTER 1. RATIONALE, HYPOTHESES AND LITERATURE REVIEW.

1.1 RATIONALE

Proper development of the placenta is critical to the success of a pregnancy. In pregnancy complications such as preeclampsia and intrauterine growth restriction (IUGR), placental development may be compromised. Processes that regulate placental growth include placental cell turnover (death and proliferation) and tissue matrix remodeling required for the formation of new blood vessels (angiogenesis). Matrix metalloproteinases (MMPs) are a group of extracellular matrix (ECM)-remodeling enzymes that play a central role in placental development, and are regulated, in part, by tissue inhibitors of metalloproteinases (TIMPs). Tissue matrix remodeling may be impaired in placentas from complicated pregnancies, leading to regions of high and low oxygen. Oxygen is a critical regulator of MMPs and TIMPs in various cell types but these effects are not well characterized in the placenta; therefore, we examined the release of MMPs and TIMPs (as indicators of tissue remodeling) from placental tissue from normal and complicated pregnancies, under conditions of high and low oxygen.

Furthermore, in pregnancies where there is inadequate uterine vascular adaptation, under-perfusion of the fetoplacental compartment may lead to secretion of factors by the placenta that alter maternal vascular reactivity, specifically, by activation of the endothelium. There is also evidence of endothelial activation in the cells of the fetoplacental compartment in preeclamptic pregnancies. MMP-2 has recently been identified as a mediator of vascular reactivity; however, its role in normal and

complicated pregnancies has not been examined. Therefore, we investigated the role of MMPs as mediators of vascular reactivity in fetal-derived human umbilical vein endothelial cells (HUVECs) from normal and preeclamptic pregnancies and in isolated vessels exposed to plasma from non-pregnant, normal pregnant and preeclamptic women.

1.2 HYPOTHESES

As mentioned in the rationale, MMPs play an important role in ECM-remodeling and can mediate vascular reactivity. I will be describing three separate projects in this thesis, each examining the role of MMPs, but in different models including placental explant cultures, endothelial cells, and isolated vessels. My general hypothesis is that *alteration in the release of MMPs and TIMPs may contribute to the pathophysiology of preeclampsia and IUGR.*

My specific hypotheses and experimental approach are described below:

Hypothesis 1: Release of MMP-2 and -9 is decreased and/or there is increased release of TIMP-2 and -1 in placental explants from pregnancies complicated by IUGR, thus contributing, in part, to impaired placental development. *Experimental approach 1*: Placental explants from normal and IUGR pregnancies are cultured at high (20%) and low (3%) oxygen, simulating placental hyperoxia and hypoxia, respectively. Levels of MMP-2 and –9 and TIMP-2 and –1 in supernatants are assessed by zymography and Western blot analysis, respectively.

Hypothesis 2: Exposure to low (<0.5% and 2%) compared to high (20%) oxygen will increase MMP-2 release from endothelial cells from normal pregnancies, thus mimicking the behaviour of endothelial cells from preeclamptic pregnancies. *Experimental approach 2:* HUVECs from normal pregnancies are cultured at low (<0.5% and 2%) and high (20%) oxygen levels and the supernatant is assessed for MMP-2 and –9 and TIMP-2 and –1, by zymography and Western blot analysis, respectively.

Hypothesis 3: Circulating factors in the plasma of women with preeclampsia enhance myogenic tone and impair endothelium-dependent relaxation in isolated arteries as compared to plasma from women with normal pregnancies, and this effect is reversed by MMP inhibition. *Experimental approach 3:* Mesenteric arteries from C57BL/6J mice are isolated and mounted on a pressure myograph to assess the effects of plasma from non-pregnant, normal pregnant and preeclamptic pregnancies in the presence and absence of MMP inhibition, on myogenic tone and endothelium-dependent vasodilation.

I will begin this thesis by describing the formation of the placenta in normal pregnancy, followed by how placental development may be impaired in complicated pregnancies. There will be a special emphasis on the critical role of matrix metalloproteinases (MMPs) in ongoing tissue remodeling within the placenta and how alterations in the levels of these enzymes may contribute to altered placental development. The next section will address the role of placental oxygen levels in early trophoblast invasion as well as in ongoing placental development, with a discussion on models of placental hypoxia, mechanisms of oxygen sensing, and the regulation of MMPs and their inhibitors by oxygen-mediated mechanisms. My focus will then be diverted to maternal and fetal vascular dysfunction in the pregnancy-specific disorder, preeclampsia, as a result of circulating factors that may be secreted from an under-perfused placenta. Finally, I will examine the circulating factors that have been implicated in the disease, followed by how

MMPs may alter vascular function in normal pregnancy and preeclampsia as a result of these circulating factors.

1.3 EARLY DEVELOPMENT OF THE HUMAN PLACENTA AND ESTABLISHMENT OF THE PLACENTAL CIRCULATION

Appropriate growth and development of the placenta is essential for a successful pregnancy. The placenta is a combination of interlocking fetal and maternal tissues that serves as the organ of exchange between mother and fetus. It is also an endocrine organ with the capacity to synthesize both steroid and polypeptide hormones (61). The human placenta is a discoid organ consisting of branched villous trees that are bathed in maternal blood. Development of the placenta begins at the time of blastocyst implantation into the maternal endometrium. The outer layer of the blastocyst is the trophoblast and the inner cell mass is the embryoblast. After the initial invasion of the endometrial epithelium, the trophoblastic cells of the blastocyst show increased proliferation and differentiate into 2 layers. The outer layer consists of trophoblast cells that fuse and form the continuous, non-proliferating, multinucleated layer called the syncytiotrophoblast (ST) that eventually surrounds the blastocyst. The remaining cellular components of the blastocyst wall, which are not in contact with maternal tissue, remain mononuclear and unfused and are called cytotrophoblasts (CTs) (11). The ST loses its capacity to divide during fusion; therefore, the CTs act as a stem cell that ensures the growth of the trophoblast by proliferation with subsequent fusion. The trophoblasts at the implantation pole (the site at which the blastocyst initially contacts the endometrium) eventually form the placenta and the rest of the trophoblasts surrounding the embryo form the smooth chorion and membranes (61).

With progressive invasion, the mass of ST increases and achieves thickness at the implantation pole, as the CTs lying underneath proliferate and fuse syncytially. Eventually, three layers are formed: the primary chorionic plate, the lacunar system and the trophoblastic shell (11). The primary chorionic plate consists of a layer of mesenchymal cells (derived from the embryoblast) that are covered by layers of CTs and ST. The lacunar system is composed of vacuoles formed by lamellae and pillars of ST called trabeculae. Within a few days, the lacunar system extends all over the blastocyst and eventually forms the intervillous space. The maternal blood eventually fills the lacunar system and moves slowly; however, with deeper invasion of the endometrium, the maternal spiral arteries are transformed into low-resistance, large-diameter arteries, resulting in higher intralacunar pressure. The transformation of the uterine vasculature is described below.

CTs invade the uterine spiral arteries and create intraluminal plugs (128) which may act as valves regulating blood flow to the intervillous space and protecting the embryo from forceful maternal blood flow. Endovascular trophoblast cells replace the endothelial layers of these vessels upon destruction of the medial, elastic, muscular, and neural tissue (74). This occurs in 2 trophoblast waves: the first is into the decidual segments of spiral arteries at 8 to 10 weeks of gestation, and the second is into the myometrial segments at 16 to 18 weeks gestation (127). By the end of the second trimester of pregnancy, the spiral arteries are lined exclusively by CTs, and endothelial cells are no longer present in the endometrial or myometrial regions. These physiologic changes create a low resistance arteriolar system and an absence of vasomotor control that allows a dramatic increase in the blood supply to the developing fetus. In fact, the spiral arteries may reach 30 times their prepregnancy diameter (15). Studies have suggested that efficient maternal blood flow in the normal human placenta is established only after the 12^{th} week of pregnancy (83, 84).

The trabecular columns of the lacunar system serve as the framework from which placental villi later develop. Shortly after the appearance of maternal erythrocytes in the lacunae, there is increased CT proliferation with subsequent fusion of the trabeculae. As a result, syncytial side branches form and protrude into the lacunae and are called primary villi. Further proliferative activity and branching of the primary villi leads to the development of primitive villous trees. Soon thereafter, mesenchymal cells derived from the extraembryonic mesenchyme invade the villi, transforming them into secondary villi. Trabeculae that maintain contact with the trophoblastic shell are not invaded with mesenchyme and are called anchoring villi. The floating villi with the fetal blood supply are extensions of stem villi originating in the chorionic plate and are the predominant sites of maternal-fetal exchange. The lacunar system is now called the intervillous space and the floating villi bring the fetal circulation in close contact with the maternal blood (11). **Figure 1.1** illustrates the structure of the placenta showing the fetal and maternal circulation and the orientation of the placental villi in the intervillous space.

During early pregnancy, the villi are still large and display a low surface area:volume ratio. There is little thinning of the trophoblast barrier that separates the maternal and fetal circulations and their diffusion capacity is limited; nevertheless, the placenta is capable of meeting fetal demands at this time. During the third trimester, fetal weight increases exponentially. In order to accommodate the demands of the fetus at this time, the placental villous tree undergoes remarkable transformations such as elaboration of terminal villi, increased vascularization, and a reduction in the thickness of the villous membrane (32). In addition, the placental barrier (consisting of ST, CT, basal lamina, connective tissue, and fetal endothelium) changes as pregnancy progresses. There is a reduction in the mean maternofetal diffusion distance from 50 – 100 μm in the second month to between 4 – 5 μm at term, in order to accommodate the increased demands of a growing fetus and ensure that sufficient quantities of oxygen and nutrients are delivered (11).

1.4 THE PLACENTA IN COMPLICATED PREGNANCIES

The critical role of the placenta means that deviations from normal development can lead to complications of pregnancy, compromising fetal growth and development. Preeclampsia is a common disorder of pregnancy [it affects 7 – 10% of all pregnancies, (136)] characterized by hypertension and proteinuria, and has widespread detrimental effects on maternal physiology (138). It is often associated with intrauterine growth restriction (IUGR) (89), characterized by suboptimal fetal growth [below the 10^{th} percentile of birthweight for gestational age (53)]. The risk of fetal death is 10 times higher in IUGR than in appropriately grown fetuses, nearly 10% of IUGR infants have physical handicaps and another 5% show a delay in mental development (102). IUGR is also associated with increased risk of adult-onset diseases such as coronary artery disease, stroke, diabetes, and hypertension (8).

The pathogenesis of these disorders begins early in pregnancy and is thought to be due, in part, to impaired trophoblastic invasion of the maternal spiral arteries; as a result, the spiral arteries keep their endothelial lining and innervation and the vessels remain narrow and reactive (21). The invasion is confined to the decidual part of the spiral arteries, while 30 - 50% of the arterioles escape endovascular trophoblast remodeling (110). Work on intrinsic defects in trophoblast function (attachment properties, adhesion molecules, enzyme secretions) has become important. Zhou et al. (178) found that in preeclampsia the invading CT expresses different adhesion molecules and integrins, and thereby fails to adapt the appropriate adhesion type. The failure of vascular remodeling prevents an adequate supply of blood from being delivered to the fetus.

While shallow trophoblast invasion and inadequate uterine vascular adaptation early in pregnancy play an important role in complicated pregnancies, the focus of my research is how ongoing tissue remodeling in the placenta may be altered. Processes that regulate placental growth include placental cell turnover (death and proliferation) and tissue matrix remodeling required for the formation of new blood vessels (angiogenesis). While placental cell turnover in complicated pregnancies has been well characterized (157, 158), the intricacies of tissue matrix remodeling are as yet understood.

A variety of placental pathologies that may be associated with impaired tissue remodeling, have been observed in pregnancies complicated by preeclampsia and IUGR including small placenta, necrosis, high degree of placental infarction, fetoplacental vasculopathy, and deficiency of the terminal villi (11, 146, 147). Matrix metalloproetinases (MMPs) are a group of extracellular matrix (ECM)-remodeling enzymes that play a central role in placental development (120) and altered levels of these enzymes and their inhibitors may contribute to impaired placental development in complicated pregnancies. The following section will provide an overview of MMPs and their inhibitors, with a special emphasis on their roles in ongoing placental remodeling.

1.5 MATRIX METALLOPROTEINASES

1.5.1 Overview

MMPs are a family of enzymes involved in remodeling of the ECM in a number of physiological and pathophysiological processes. In normal physiology, MMP activity is associated with ovulation (26), trophoblast invasion (72) and angiogenesis (31), whereas, unregulated MMP activity has been implicated in rheumatoid arthritis and osteoarthritis

(29), tumour invasion (101) and degradation of the myelin-basic protein in neuroinflammatory diseases (30).

MMPs are secreted as partially active zymogens that require proteolytic cleavage (115, 159) or reconfiguration (by biological molecules *in vivo*) (106, 124, 126) of the N-terminal pro-peptide for full activation. The zymogens (pro-MMPs) consist of three discernible domains: the autoinhibitory domain, the zinc-binding catalytic domain, and the hemopexin-like C-terminal domain (159). The sequence PRCGVPD in the autoinhibitory domain is thought to be involved in maintaining the inactive form of the enzyme by coordinate binding of cysteine thiolate and the zinc ion, which protects the active site from substrates (16). Once the cysteine-zinc interaction is interrupted, catalytic activity is restored. This is known as the cysteine switch activation mechanism. MMPs are active at neutral pH and require calcium (Ca^{2+}) for full activity. Pertinent to our studies, MMP-2 and MMP-9 are gelatinases produced by many cell types including endothelial cells (16) and trophoblasts (113, 120), and degrade basement membrane collagens.

The regulation of MMPs is complex and occurs at multiple levels including gene activation and transcription, translation and secretion of the latent enzyme, proenzyme activation and inactivation by endogenous inhibitors (16). *In vivo*, MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs), which interact with the proenzyme at the amino terminus, the region flanking the enzyme active site (18, 56). The resulting steric hindrance effectively blocks the MMP active site from binding to components of the ECM. Under conditions of stasis, there is an equimolar ratio of MMPs and TIMPs outside the cell, with one TIMP binding to one MMP, resulting in a lack of proteolytic activity and stable cell populations (98). During cell proliferation and migration, however, there must either be a shift in the relative levels of MMPs and TIMPs or a dissociation of TIMPs from MMPs. TIMP-1 inhibits the active form of all

MMPs and the latent form of MMP-9 (pro-MMP-9) and is the most widely distributed TIMP (71). TIMP-2 binds to both the inactive and active forms of MMP-2, while its inhibitory effect over the other MMPs is significantly lower (161).

Interestingly, TIMP-2 also mediates the activation of pro-MMP-2 through the formation of a trimolecular complex between membrane-type MMP (MT1-MMP), TIMP-2 and pro-MMP-2 (87). In these complexes, the C-terminal tail of TIMP-2 interacts with the C-terminal hemopexin domain of pro-MMP-2, and the N-terminal inhibitory domain of TIMP-2 binds to the catalytic site of MT1-MMP. A nearby free MT1-MMP then activates pro-MMP-2 by initiating a cleavage in the pro-peptide domain of pro-MMP-2, generating the 62kD intermediate form. This partially active MMP-2 is further processed to the 59kD fully active form by autocatalysis. TIMP-2 allows activation to occur as long as its concentration does not exceed the binding capacity of MT1-MMP (119). **Figure 1.2** illustrates the activation of pro-MMP-2 on the surface on endothelial cells.

1.5.2 The importance of MMPs in ongoing placental development

MMPs are vital during angiogenesis, a process by which new blood vessels develop from the existing microvascular bed. Investigators have demonstrated that endothelial tube formation is blocked by MMP inhibitors (75, 114). Furthermore, TIMPs have been shown to prevent endothelial cell migration and tube formation (1). The processes that are involved in remodeling of the fetomaternal interface are improperly understood but may likely involve MMPs and their ability to remodel the ECM, a necessary step for cell migration in angiogenesis. Indeed, MMP-2 and MMP-9 are expressed in invasive extravillous trophoblasts and in the human placenta (82). Niu et al. (120) reported that conditioned media of human placental culture contained the inactive and active forms of MMP-2 and MMP-9 as well as their inhibitors TIMP-2 and TIMP-1. Furthermore, these

enzymes were also detected in placental tissue extracts indicating ongoing production within the placenta. Riley et al. (133) suggested that MMP-2 and MMP-9 are likely to be involved in tissue remodeling in the ovine placenta, contributing to its increasing complexity as gestation progresses. Collectively, the *in vitro* studies suggest that ongoing placental growth and development may depend on a balance between the secretion of MMPs and TIMPs by trophoblasts (73) and placental fibroblasts (129).

Ongoing placental development in pregnancy depends on a delicate balance of factors. Local oxygen environment is an important factor that must be considered as it can have a profound impact on the differentiation of the placental terminal villi (92). The following section will describe the importance of oxygen in early gestation, models of placental hypoxia, mechanisms of oxygen sensing, and the regulation of MMPs and TIMPs by changes in oxygen tension.

1.6 EFFECTS OF OXYGEN ON PLACENTAL DEVELOPMENT

1.6.1 Oxygen levels in early gestation

In early gestation (8 weeks), the mean oxygen pressure of the intervillous space is estimated to be ~ 22 mmHg, whereas at 12 weeks gestation, this value is ~ 55 mmHg (141). *In vitro* studies have shown that early in gestation, low oxygen tension stimulates proliferation of CTs and inhibits their differentiation along the invasive pathway and may explain why the mass of the placenta increases more rapidly than that of the embryo (68). After the first 12 weeks of pregnancy the intervillous space switches from a relatively hypoxic to an enhanced oxygen environment and may contribute to a switch to the invasive trophoblast phenotype for the second wave of invasion in to the maternal spiral

arteries. This may also explain why these cells extensively invade the arterial (higher oxygen tension) rather than the venous side of the uterine circulation.

1.6.2 Models of placental hypoxia

Oxygen levels also play an important role in ongoing placental development. Oxygen is a major regulator of the angiogenic factors vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF); hypoxia has been demonstrated to upregulate VEGF expression (116, 155, 174) but downregulate PlGF expression (154). The balance between these two factors may be important in ongoing placental development. For example, Cooper et al. (39) reported that VEGF mRNA levels were significantly lower in placentas from preeclamptic pregnancies and Khaliq et al. (90) demonstrated that mRNA and protein levels of PlGF were increased in placentas from IUGR pregnancies. Detailed ultrastructural studies in placentas from complicated pregnancies (95, 105) have described three distinct types of hypoxia that may occur in the fetoplacental unit and influence placental development (92). Preplacental hypoxia describes a situation where the mother, placenta and fetus are hypoxic, as in pregnancy at high altitude, maternal anaemia and cyanotic maternal cardiac diseases. Uteroplacental hypoxia describes a situation where maternal oxygenation is normal, but because of impaired uteroplacental circulation, the placenta and fetus are hypoxic. Finally, postplacental hypoxia describes the situation where the mother is normoxic, and the placenta, because of reduced oxygen extraction by the fetus, shows higher oxygen levels than normal. This situation is also described as placental hyperoxia and refers to the oxygen tension in the intervillous space being closer to maternal arterial oxygen levels. Interestingly, placentas from each of these situations of fetoplacental hypoxia demonstrate differences in angiogenesis, relative levels of VEGF and PlGF, and morphology of placental villi (91). Taken together, these findings suggest that cellular oxygen plays a crucial role in placental development. Yet

the question remains, how are placental cells able to sense the oxygen level in their local environment?

1.6.3 Mechanisms of Oxygen Sensing

Much controversy exists regarding oxygen sensing and the mechanism among various cell types and between different species (24). An oxygen-sensing-signalling pathway must include an oxygen sensor with the ability to detect changes in ambient oxygen levels and initiate a signal transduction pathway leading to activation/deactivation of specific effectors (50). Transcription factors such as hypoxia inducible factor (HIF)-1, activating protein (AP)-1, and nuclear factor kappa B (NF-κB) constitute effectors that are regulated by hypoxia.

HIF-1 is a heterodimeric complex consisting of an α-subunit and β-subunit. The expression of both subunits is up-regulated by hypoxia and the proteins degrade rapidly upon re-oxygenation (150). HIF-1 regulates the transcription of hypoxia-inducible genes, such as erythropoietin and VEGF (152) through protein phosphorylation and/or modulation of redox potential (24).

Modulation in the DNA-binding activities of the transcription factor AP-1 has been reported in response to anoxia in tumour cell lines, possibly through the *c-jun* and *c-fos* genes. c-Jun and c-Fos (the products of the *c-jun* and *c-fos* genes), constitute the AP-1 transcription factor itself, and contain conserved redox-sensitive cysteine residues in their DNA-binding domains; therefore, changes in redox potential can regulate AP-1 activity (55).

NF-κB is a trimer composed of two DNA binding proteins and an inhibitory subunit (IκB), which keeps the complex sequestered in the cytosol in an inactive form. NF-κB is activated by the dissociation of IκB from the complex, allowing the DNA binding dimer to travel to the nucleus, where it can rapidly induce the expression of a number of genes (24). Rapid activation of NF-κB has been demonstrated in cells exposed to hypoxic conditions (94). Hypoxic stimuli lead to the degradation of the IκBα chaperone protein through phosphorylation of tyrosine residues (10, 94), allowing nuclear translocation and binding of the complex to elements in the promoters of specific genes, leading to transcriptional activation.

Many putative oxygen sensors and oxygen-sensing mechanisms have been proposed in mammalian cells and include roles for heme-containing proteins, mitochondria (through oxidative phosphorylation), reactive oxygen species, and NAD(P)H oxidase (50, 151). In endothelial cells, oxygen appears to signal through a heme-containing sensor because inhibitors of heme biosynthesis blunt the hypoxic response (55). Furthermore, transition metals that can substitute for iron in the heme structure (including cobalt and nickel), but which are incapable of binding oxygen, render the sensing molecule unresponsive to oxygen tension (55). HIF-1, AP-1 and NF-κB are activated in endothelial cells as a function of the oxygen tension (7). De Marco et al. (50) suggested the involvement of heme-containing proteins and/or mitochondria as oxygen sensors in trophoblasts, which then initiate HIF-1 activation and subsequent expression of hypoxia-inducible genes responsible for trophoblast differentiation. Human placental NAD(P)H oxidase may also function as an oxygen sensor in the placenta, but perhaps only at a later stage in gestation (108).

As preeclampsia is associated with shallow extravillous trophoblast invasion into the maternal spiral arteries, it is likely that there is a potential failure of trophoblasts to sense changes in oxygen during early placental development. With regard to ongoing placental remodeling, the ability of placental cells to sense changes in oxygen levels may affect their MMP/TIMP secretory profiles. The following section will address the mechanisms by which levels of MMPs and TIMPs may be altered by changes in oxygen as well as the ability of various cells types to alter their production and release of these enzymes in response to changes in oxygen tension.

1.6.4 Oxygen regulation of MMPs and TIMPs

The genes encoding MMPs and TIMPs must contain oxygen-sensitive elements in order for changes in enzyme expression to occur under conditions of varying oxygen. The MMP-9 promoter contains both AP-1 and NF-κB sites that enable its expression to change under varying oxygen conditions (81). The existence of an oxygen-sensitive region in MMP-2 was initially disputed (164); however, recently, an AP-1 binding site has been reported (13). A hypoxia response element, containing a HIF-1 binding motif, has also been identified in the TIMP-1 promotor (121).

Investigators have demonstrated numerous effects of oxygen tension on the release of MMPs and their inhibitors in various cell types; however, the effects of oxygen tension on cells of the fetoplacental compartment have not been well characterized. Release of MMP-2 has been shown to increase in human macrovascular endothelial cells (12), decrease in proximal tubular epithelial cells and endometrial stromal cells (125, 177) and remain unchanged in trophoblast and breast cancer cells (28) when exposed to low oxygen tensions. MMP-9 release was increased in MDA-MB-231 breast cancer cells but

decreased in MCF-7 breast cancer cells (28), and remained unchanged in neuroblastoma cell lines (81) in response to conditions of low oxygen. Reports on oxygen-mediated TIMP-1 release are equally diverse. Decreased TIMP-1 expression has been reported in human breast cancer cells (28) and osteoblasts (172) in response to conditions of hypoxia; however, others have shown increased levels in human renal fibroblasts (121), proximal tubular epithelial cells (125) and human microvascular endothelial cells (145). It is important to note that MMP/TIMP release in response to varying oxygen levels depends on the tissue and cell type, as well as the duration and direction of the change in oxygen environment compared to the physiological level.

Low oxygen levels within ischemic regions of a hypoperfused placenta may lead to alterations in MMP production and release, resulting in impaired placental development. Furthermore, an underperfused placenta is capable of secreting factors that have widespread effects in the maternal circulation, as in the pregnancy-specific disorder, preeclampsia. The following section will focus on a discussion of maternal vascular effects in preeclampsia.

1.7 MATERNAL VASCULAR PATHOPHYSIOLOGY OF PREECLAMPSIA

Despite decades of extensive investigation, the etiology and pathophysiology of preeclampsia remain poorly understood (63, 103, 134, 138). Maternal constitutional factors such as genetic predisposition, immunological maladaptation to pregnancy and pre-existing vascular diseases have been implicated in the etiology of the disease (167). Traditionally, preeclampsia has been classified as a hypertensive disease of pregnancy; nevertheless, it has widespread detrimental effects, with hypertension representing only one manifestation. Pathological changes are observed in blood volume, coagulation, liver, kidney, and cerebral arteries (138).

Many of the pathophysiologic changes associated with preeclampsia are essentially a failure of the adaptations seen in normal pregnancy. Increase in plasma volume is smaller or non-existent (23, 79); hematocrit is commonly elevated (33); systemic vascular resistance is elevated resulting in elevated blood pressure (104); perfusion is reduced to several organs (135); and there is an increased pressor response to the vasoconstrictor, angiotensin II (67). The cause of the vasospasm and increased sensitivity to circulating pressor agents is not clear, although numerous factors have been implicated. One of these factors may be the ability of the vascular endothelium to produce vasodilators and vasoconstrictors. In preeclampsia, there may be a change in the balance between endothelial-derived vasoconstrictors and vasodilators, such that the action of vasoconstrictors is favoured, overall (89).

For example, while normal pregnancy is associated with increased synthesis of the vasodilator, nitric oxide (2, 38) and increased expression and activity of nitric oxide synthase in the human uterine artery (118), alterations in nitric oxide synthesis have been reported in women with preeclampsia (40). Normal pregnancy is also associated with increased urinary excretion of 6-keto-prostaglandin $F_{1\alpha}$, a hydration product of the vasodilator prostacyclin (prostaglandin I_2) (176), whereas in preeclampsia, there are reports of alterations in the production of prostacyclin (60). Endothelium-derived hyperpolarizing factor has been shown to mediate relaxation in small mesenteric arteries of pregnant rats (69); however, its role in preeclampsia has not been investigated. Finally, the production of vasoconstrictors such as endothelin (51) and thromboxane A_2 (170) are increased in women with preeclampsia. **Figure 1.3** summarizes the vasodilators and vasoconstrictors released from the endothelium that may be involved in normal pregnancy and preeclampsia.

Changes in the levels of vasoconstrictors and vasodilators from the endothelium may indicate a perturbation in the function of the endothelium itself. Preeclampsia is characterized by widespread vascular endothelial dysfunction that may be caused by circulating factors released by a hypoxic placenta. The following section will focus on the role of the endothelium in normal physiology and in the pathophysiology of preeclampsia.

1.8 ENDOTHELIAL DYSFUNCTION IN PREECLAMPSIA

1.8.1 The endothelium in normal physiology

The endothelium was traditionally viewed as an inert membrane lining the circulatory system, its primary function being the maintenance of vessel wall permeability (34); however, it is, in fact, a complex and active organ with metabolic, endocrine and structural functions. Endothelial cells exist in a continuous monolayer and line vessels in every organ system, regulating the flow of nutrient substances, biological molecules and blood cells. The presence of membrane-bound receptors enables the endothelium to facilitate the transport of selective molecules. The endothelium also plays an important role in regulating blood flow by generating an anti-thrombotic surface through which plasma and cellular components can be transported throughout the vasculature. Blood flow is also regulated in part through the secretion and uptake of vasoactive substances by the endothelium that act in a paracrine manner to constrict and dilate specific vascular beds (34). Normal vascular endothelial cells produce materials that buffer responses to pressor agents, and include nitric oxide, prostacyclin, and endothelim-derived hyperpolarizing factor. The endothelium appears to be altered in pregnancy, resulting in

vasodilation either as a result of increased release of vasodilators or a decreased vasoconstrictor output (130).

1.8.2 Maternal endothelial activation in preeclampsia

As reviewed by Roberts et al. (140), the multi-system effects of preeclampsia may be explained by endothelial dysfunction initiated by a factor (s) produced by the placenta as a consequence of reduced trophoblastic invasion and reduced blood flow. If the normal functions of the endothelium are disrupted, pathophysiological changes occur including increased sensitivity to pressors, activation of the coagulation cascade, and loss of vascular integrity, all characteristic of preeclampsia. Injured endothelial cells express new functions (143) including increased production of vasoconstrictors such as endothelin (175) and procoagulants such as factor XII activator and tissue factor (142), decreased expression of anticoagulant activity, and enhanced platelet activation and adhesion (142).

There is a growing body of data demonstrating morphologic, functional and biochemical evidence of endothelial injury in preeclampsia. Preeclampsia has features in common with thrombotic thrombocytopenic purpura and hemolytic uremia syndrome, disorders in which endothelial dysfunction is thought to be pathogenetically important (138). Morphologic changes of the endothelium are reported in the kidneys, spiral arteries, liver, and umbilical arteries (63). Functional evidence of altered endothelial cell permeability is demonstrated by the increased disappearance rate of Evans blue dye from the vascular compartment in preeclampsia (27). Biochemical evidence supports the existence of factors released by activated endothelial cells including von Willebrand factor (52, 62), endothelin (163), and the cellular epitope of fibronectin (162). There is also a change in balance between tissue plasminogen activator and inhibitor and between prostacyclin and thromboxane A_2 (134).

1.8.3 Evidence of endothelial activation in the fetoplacental compartment of pregnancies complicated by preeclampsia

The vast evidence supporting maternal endothelial activation in preeclampsia leads to the question of whether endothelial activation is also present in the fetoplacental compartment. In fact, much evidence supports the idea that fetal-derived cells from preeclamptic pregnancies demonstrate altered endothelial function, suggesting that this phenomenon is not limited to the maternal endothelium alone. It was recently reported that fetal-derived HUVECs from preeclamptic pregnancies have altered cation membrane permeability and activity of the endothelial nitric oxide synthase pathway (160). Similarly, Wang et al. (169) reported increased endothelial cell permeability in preeclamptic HUVECs, possibly reflecting disorganized and diminished expression of endothelial cell junctional proteins. A number of other investigators have reported marked morphological abnormalities (41, 49, 70) and alterations of passive and active mechanical properties (14) in umbilical vessels of preeclamptic women compared to women with uncomplicated pregnancies. The cells from the umbilical vessels also demonstrate abnormal production and release of factors such as fibronectin (49) and nitric oxide and prostacyclin (132). Collectively, the data imply that the fetal-derived cells of the umbilical cords from preeclamptic pregnancies demonstrate altered endothelial function.

It is clear that maternal endothelial cells, as well as fetal-derived cells, are activated in preeclampsia. The activation of the endothelium may contribute to altered maternal vascular reactivity in preeclampsia. This may be due to intrinsic changes in the maternal vasculature, factors in the maternal circulation that affect the vasculature, or a combination of both. The following section will examine the evidence for maternal

vascular dysfunction in preeclampsia. It will begin with a discussion of how vessels function in normal physiology, and how they regulate blood flow through the vascular myogenic response. Signal transduction mechanisms associated with the vascular myogenic response will also be examined. The focus will then turn to how isolated vessels from preeclamptic pregnancies are fundamentally different from those from normal pregnancies, with a specific emphasis on their ability to elicit a myogenic response.

1.9 EVIDENCE OF MATERNAL VASCULAR DYSFUNCTION IN PREECLAMPSIA

1.9.1 Resistance vessels and smooth muscle in normal physiology

Blood flow and arterial blood pressure are primarily regulated by small arteries and arterioles that are known as resistance vessels. Smooth muscle fibers are the main component of the walls of the resistance vessels and undergo synchronous activity, that is, the whole muscle responds to stimulation as a single unit. This phenomenon occurs through gap junctions, through which action potentials occurring in one cell are propagated to other cells (165). Parts of endothelial cells also project into the smooth muscle layer (myoendothelial junctions) at various points, suggesting a functional interaction between the endothelium and adjacent vascular smooth muscle.

The sliding filament mechanism is believed to mediate vascular smooth muscle contraction (179). The actin filaments in smooth muscle do not have the calcium (Ca^{2+})-binding protein troponin that mediates Ca^{2+}-triggered cross-bridge activity in both skeletal and cardiac muscle. Instead, cross-bridge cycling in smooth muscle is controlled

by a Ca^{2+}-regulated enzyme that phosphorylates myosin, which is then able to bind to actin. The following sequence of events occurs after a rise in cytosolic Ca^{2+} (from intracellular and extracellular sources) in a smooth muscle fiber: (1) Ca^{2+} binds to calmodulin (CaM), a calcium-binding protein whose structure is similar to troponin; (2) the Ca^{2+}-CaM complex binds to a protein kinase, myosin light-chain kinase (MLCK), thereby activating the enzyme; (3) the active protein kinase then uses adenosine triphosphate (ATP) to phosphorylate myosin light chains in the globular head of myosin; (4) phosphorylated myosin binds to actin filaments, resulting in contraction. Relaxation occurs when myosin is dephosphorylated, a reaction mediated by myosin light chain phosphatase (MLCP) (165).

1.9.2 Local control of blood flow – the vascular myogenic response

One of the mechanisms that regulates blood flow is the vascular myogenic response. This describes the phenomenon that blood vessels respond to elevations in transmural pressure with constriction, and to pressure reduction with dilation. This property is inherent to smooth muscle and is independent of neural, metabolic and hormonal influences (46). The myogenic response is most pronounced in arterioles but can be occasionally demonstrated in arteries, venules, veins and lymphatics. An inverse relationship between vessel size and myogenic responsiveness is consistently observed among arterioles of a given vascular network (45).

Figure 1.4 illustrates the myogenic response of a cannulated arteriole to a step increase in pressure in the presence of extracellular Ca^{2+} (active). After the increase in pressure, an initial, passive distension is followed by constriction. The absence of extracellular Ca^{2+} abolishes vascular myogenic tone, as depicted by the passive curve.

Two of the most important functions of the vascular myogenic response are the establishment of basal vascular tone and autoregulation of blood flow and capillary hydrostatic pressure. Basal vascular tone establishes an underlying arteriolar constriction upon which other control mechanisms produce vasodilation or vasoconstriction. The local regulation of blood flow protects capillary beds from large increases in hydrostatic pressure induced by postural changes (47). Although the exact signal transduction mechanisms underlying myogenic tone remain uncertain, it is clear that the phenomenon resides within the vascular smooth muscle cell and can be modulated by a variety of endothelial and neurohumoral factors (46).

1.9.3 Signal transduction mechanisms underlying the myogenic response

A plethora of transduction mechanisms have been implicated in the vascular myogenic response; the precise mechanism is unclear and is likely to be very complex. Evidence suggests that the myogenic response may reflect an improved excitation-contraction coupling resulting from membrane depolarization and increased Ca^{2+} permeability. Investigations have supported the role of vascular smooth muscle depolarization, mechanosensitive channels, nonselective cation channels, potassium, chloride, and voltage gated Ca^{2+} channels in the mediation of the myogenic response. Furthermore, the function of carrier-mediated ion exchangers and transporters on vascular smooth muscle plasma membranes may also be modulated by membrane stretch in response to increased pressure (46). Other mechanisms that have been studied in regard to the myogenic response in arterioles include propagation through gap junctions, control of MLC-phosphorylation, the role of the smooth muscle binding proteins caldesmon and calponin, protein kinase C, G-proteins, the adenylate cyclase and arachidonic acid pathways, and more recently, the role of the cytoskeleton and extracellular matrix (46).

The role of Ca^{2+} as a second messenger has been extensively studied in the myogenic contraction. The advent of Ca^{2+} sensitive fluorescent dyes together with video-based imaging and photometer techniques has allowed the study of Ca^{2+} dynamics in resistance vessels. Studies have demonstrated the importance of both extracellular and intracellular sources of Ca^{2+} in eliciting the myogenic response. Specific Ca^{2+} influx/efflux mechanisms are extremely complex and further investigations are warranted to fully understand Ca^{2+} dynamics in arterioles (80).

Due to the sheer complexity of mechanisms involved in eliciting the vascular myogenic response and the abundance of conflicting data, further studies are necessary to delineate the ways in which these mechanisms work in an integrated manner.

1.9.4 The role of the endothelium in the myogenic response

The possibility that vascular endothelial cells might be required for normal myogenic responsiveness was suggested by the experiments of Harder (76). His findings supported the role of the endothelium in eliciting the myogenic response and were confirmed by Katusic et al. (86) who found that endothelium removal from canine basilar arterial rings prevented the active force development by stretch. Meininger and Davis (111) suggested that vascular wall stretch or distension induces the release of an endothelium-derived vasoconstrictor or decreases the release of an endothelium-derived relaxing factor. However, subsequent studies on various types of blood vessels have demonstrated, rather convincingly, that the endothelium is not required for myogenic responsiveness (111), suggesting that earlier data supporting a role for the endothelium can be explained by vascular smooth muscle damage resulting from chemical denudation methods. The role

of the endothelium in myogenic responsiveness is controversial and suggests that there may be species- or tissue-specific differences.

1.9.5 Myogenic tone in pregnancy

There is conflicting evidence regarding the effect of pregnancy on myogenic tone. Some studies have demonstrated reduced myogenic tone, modulated by endothelium-derived nitric oxide, in mesenteric arteries (112) and small renal arteries (65) from pregnant rats. Similarly, Veerareddy et al. (168) demonstrated reduced myogenic responses, also modulated by nitric oxide, in the mesenteric and main uterine arteries in late pregnant compared with non-pregnant mice. Others (35, 100), however, have reported no change of myogenic tone in mesenteric arteries obtained from late pregnant rats. As preeclampsia is a condition characterized by altered vascular hemodynamics, it is possible that an abnormality of the myogenic response in resistance vessels may contribute to impaired vascular blood flow.

1.9.6 Myogenic tone in preeclampsia

Although a few studies have reported blunted relaxation to endothelium-dependent vasodilators in isolated arteries from preeclamptic pregnancies (3, 93, 109), very few studies have investigated whether myogenic tone is altered in preeclampsia. VanWijk et al. (166) studied myogenic responses in subcutaneous arteries from normal and preeclamptic pregnancies and reported no differences between the two groups. Kublickiene et al. (96) also reported that the degree of myogenic tone at different pressure steps in myometrial arteries from women with normal and preeclamptic

pregnancies was similar. Interestingly, vessels from preeclamptic pregnancies had a significantly increased distensibility index (i.e. increase in diameter as a function of pressure) compared to vessels from normal pregnancies, suggesting a compensatory response in preeclampsia to preserve blood flow in the uteroplacental vascular bed. As the vascular smooth muscle is integral to the myogenic response, VanWijk et al. (166) addressed vascular smooth muscle reactivity in preeclamptic compared to normal pregnancies and reported that vascular smooth muscle Ca^{2+} sensitivity is increased in vessels from preeclamptic pregnancies. Moreover, *in vivo*, vascular smooth muscle function is under the influence of the sympathetic nervous system and interestingly, sympathetic nervous activity is decreased in normal pregnancy and increased in preeclampsia (149).

Indeed, there is mounting evidence that supports the fact that isolated vessels from the maternal vasculature of women with preeclampsia behave differently from those from the vasculature of women with normal pregnancies. Long-term exposure of the maternal vasculature to circulating factors may be one of the causes of this altered vascular reactivity. This concept has been addressed through *in vitro* studies using isolated cells and vessels from normal pregnancies that are exposed to the plasma of women with preeclampsia. The following section will describe the candidate factors that have been implicated in preeclampsia, how they affect vascular function and, finally, how these factors may stimulate the release of MMPs from vessels, causing changes in vessel function.

1.10 CIRCULATING FACTORS IN PREECLAMPSIA

1.10.1 In vitro studies

Evidence that terminating the pregnancy, and more specifically, that delivery of the placenta results in recovery, suggests that a poorly perfused placenta may be the origin of production of the factor(s) that causes endothelial dysfunction. The circulating factor hypothesis has been investigated primarily in a number of *in vitro* studies that have compared the effects of serum or plasma samples taken before and after delivery from normal pregnant and preeclamptic women, on the function of cultured endothelial cells. Rodgers et al. (143) demonstrated that cytotoxicity in cultured HUVECs increased following incubation with serum from women with preeclampsia when compared to normal pregnant controls. It was also noted that the cytotoxic effect of serum collected pre-delivery was greater than that obtained post-delivery. Roberts et al. (139) subsequently found that serum from women with preeclampsia caused a greater release of chromium-labeled chromate (an indicator of lethal cell damage) from HUVECs than serum from normal pregnant women. With additional investigations the group concluded that the circulating factor induced an alteration in endothelial function, rather than gross morphologic injury and cell death as initially thought (137).

There is a surprising paucity of information pertaining to the effects of plasma from women with preeclampsia on vascular function in isolated vessels. Studies have reported that plasma from women with preeclampsia leads to impaired endothelium-dependent relaxation in vessels form normal pregnancies (4, 66, 77). One study has addressed the effects of plasma from women with preeclampsia on myogenic tone in vessels from normal mice, and reported that myogenic tone was significantly enhanced in these vessels (66). It is obvious from these observations that factors contained in the plasma of women with preeclampsia alter the vascular reactivity of normal vessels; nevertheless, there are few studies that have attempted to identify these factors.

1.10.2 Identity of the circulating factor

Preliminary studies to identify the circulating factor(s) reported that the factor is susceptible to heat treatment and is separated by ammonium sulphate precipitation, suggesting a protein component (78). Furthermore, the activity of plasma from women with preeclampsia is partially removed by charcoal precipitation, suggesting a possible lipid component (78). Davidge et al. (43) provided evidence for the presence of two distinct factor groups in plasma from women with preeclampsia. A high molecular-weight factor stimulated production of nitric oxide from microvascular endothelial cells whereas an aqueous factor of low molecular weight stimulated release of prostacyclin. More recently, matrix metalloproteinase (MMP)-2 has been implicated as a vasoactive mediator, whose levels are elevated in the plasma of women with preeclampsia (117). Brockelsby et al. (19) demonstrated that co-incubation of vessels from normal pregnancies with plasma from women with preeclampsia and a neutralizing antibody to VEGF subsequently reversed the impaired relaxation observed when vessels were incubated in plasma alone, suggesting that circulating VEGF may be the culprit. Similarly, Gandley et al. (66) reported reversal of impaired relaxation when mesenteric vessels from mice were co-incubated in the plasma of women with preeclampsia and an AT_1 receptor (receptor for angiotensin II) antagonist, suggesting that circulating angiotensin II may be involved in the pathophysiology of preeclampsia. Indeed, circulating levels of VEGF are elevated in the plasma (153) and serum (6, 20) of women with preeclampsia. Similarly, despite reduced circulating levels of angiotensin II (22, 99), preeclampsia is associated with an increased pressor response to this vasoconstrictor (67). Other circulating factors that may contribute to the pathophysiology of preeclampsia include the cytokine tumor necrosis factor-α (97) and maternal triglycerides (171, 173), the levels of which are elevated in preeclampsia.

1.10.3 Effects of circulating factors on endothelial release of vasoconstrictors and vasodilators

Circulating factors may possess the ability to alter the production and release of vasodilators and vasoconstrictors from the endothelium in preeclampsia. Numerous studies have examined the production of the vasodilators prostacyclin and nitric oxide in cultured endothelial cells. Paradoxically, although preeclampsia is associated with deficient intravascular production of prostacyclin (25) and possibly nitric oxide (44), investigators have reported increased *in vitro* production of prostacyclin and nitric oxide from endothelial cells exposed to the plasma of women with preeclampsia compared to plasma of normal pregnant women (5, 42, 48). The explanation for this paradox is unclear but may include compensatory up-regulation of effectors with down-regulation of responsiveness, differences in concentration of effectors *in vitro* and *in vivo*, or the possibility that there are other sources of nitric oxide other than the endothelium. Alternatively, increased levels of nitric oxide in the face of oxidative stress decreases the bioavailability of nitric oxide through the production of peroxynitrite, a powerful oxidant that may alter vascular function (9). In fact, there is evidence of increased peroxynitrite formation in the vasculature of women with preeclampsia (144). Elevated levels of the vasoconstrictor, endothelin-1, have been reported in the plasma of women with preeclampsia (163); nevertheless, endothelin-1 release has been found to be increased (148), decreased (17), or unchanged (64) in endothelial cells exposed to the plasma of women with preeclampsia compared to plasma from normal pregnant women.

Evidence provided by *in vitro* studies suggests that circulating factors contained in the plasma of women with preeclampsia have the ability to alter the balance between vasodilators (such as prostacyclin and nitric oxide) and vasoconstrictors (such as endothelin) released from the endothelium, and these pathways may contribute, in part, to the pathophysiology of preeclampsia.

1.10.4 The role of MMPs in vascular function

An emerging vascular pathway of interest involves MMPs, specifically, MMP-2, the levels of which are elevated in the plasma of women with preeclampsia (117). In addition to its traditional role in ECM-remodeling, MMP-2 can mediate vascular reactivity. Circulating factors such as VEGF (107, 117), tumor necrosis factor-α (54, 131) and angiotensin II (36) are capable of stimulating the production and/or release of MMPs. The role of MMPs as mediators of vascular function in preeclampsia is presently unknown.

Studies have shown that MMPs may be involved in mediating vasoconstriction of the vasculature. Fernandez- Patron et al. (58) found that MMP-2 can cleave big endothelin at the Gly^{32}-Leu^{33} bond, yielding the potent vasoconstrictor medium endothelin. Endothelin peptides may then bind to ET_A receptors on vascular smooth muscle to elicit vasoconstriction, or to ET_B receptors on the vascular endothelium to mediate vasodilation (156). In a subsequent study, pharmacological inhibition of MMP-2 induced endothelium-independent vasorelaxation in rat mesenteric arteries. Further investigations showed that MMP-2 cleaved calcitonin gene-related peptide specifically at the Gly^{14} – Leu^{15} peptide and reduced its vasodilatory potency by 20-fold (59).

Evidence also supports that MMPs may be mediators of vasodilation. The serine protease thrombin induced long-lasting vasodilator effects associated with a dose-dependent release of vascular MMP-2 in rat mesenteric arteries (57). These vasodilator effects were mimicked by an infusion of recombinant MMP-2 but were inhibited by TIMP-2 and an MMP-2-specific antibody. More recently, the role of MMPs in pregnancy has been

investigated and also supports their role as vasodilators. Novak et al. (122) showed that specific and non-specific MMP inhibitors reversed the reduced myogenic reactivity associated with pregnancy. Relaxin, a circulating hormone that mediates vasodilation in the renal vasculature (123), has been found to upregulate MMP-2 in small renal arteries (88) and subsequent MMP inhibition abolishes the relaxin-induced vasodilation and hyperfiltration (85). The mechanism by which MMPs can influence vasodilation is not clearly understood, but studies have supported the role of endothelin, through the ET_B receptor pathway, as a mediator of renal vasodilation and hyperfiltration in chronically instrumented pregnant rats (37) and as a mediator of reduced myogenic reactivity in small renal arteries from pregnant rats (65). Since MMP-2 can cleave big endothelin to generate endothelin-peptides, it is possible that one or more of these peptides may act on the ET_B pathway to mediate vasodilation.

The role of MMPs in mediating endothelium-dependent relaxation and the vascular myogenic response has not been investigated. Furthermore, circulating factors in the plasma of women with preeclampsia may stimulate the release of MMPs from the vasculature, and alter vascular reactivity. This novel and intriguing possibility provides a new perspective on the role of MMPs in the pathophysiology of preeclampsia.

1.11 SUMMARY

Preeclampsia and IUGR are significant complications of pregnancy that may be associated with impaired tissue remodeling of the placenta. Furthermore, shallow trophoblast invasion and inadequate transformation of the uterine vasculature early in pregnancy, may lead to the development of an under-perfused fetoplacental unit. In turn, the placenta may release factors that cause activation of the maternal endothelium, which ultimately results in impaired vascular reactivity. MMPs have a diverse range of

biological functions, traditionally in ECM-remodeling and recently, in the regulation of vascular activity. A shift in the balance between MMPs and their inhibitors may have severe implications on placental remodeling and vascular reactivity, and may contribute the pathophysiology of preeclampsia.

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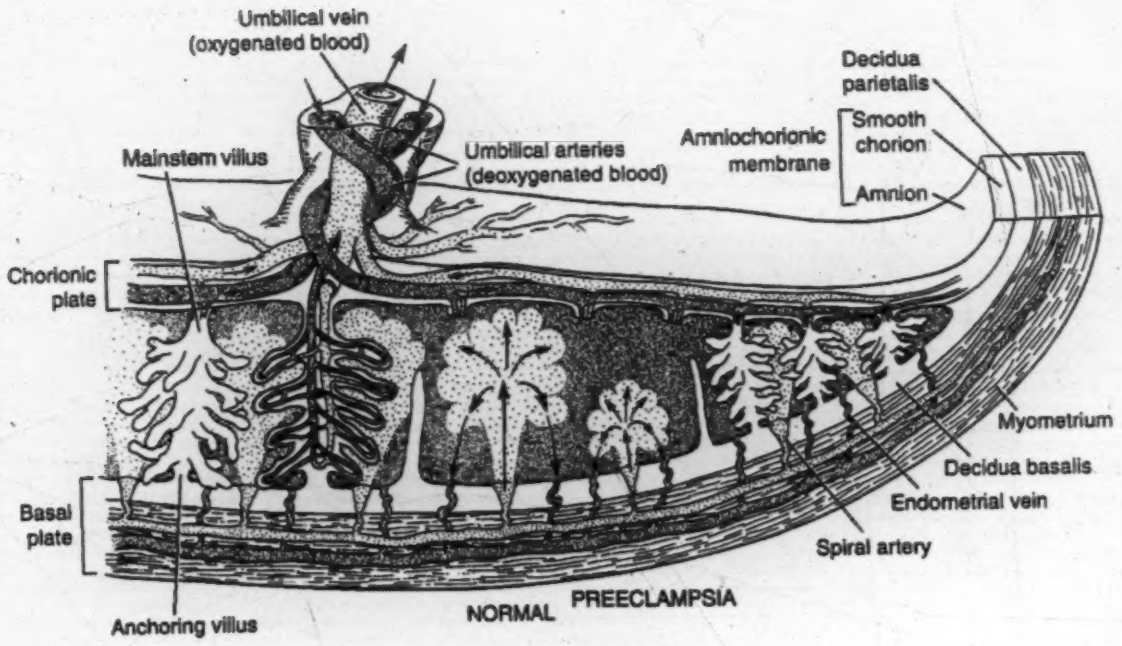

Figure 1.1 The structure of the human placenta.

The diagram illustrates the chorionic plate (contacts the fetal circulation), the basal plate (contacts the maternal circulation), umbilical vein, umbilical arteries, and the structure of the placental villi in the intervillous space. The maternal spiral arteries transport oxygenated blood to the intervillous space where it is absorbed by the umbilical vein and transported to the fetus. The umbilical arteries remove wastes from the fetus, and transport them to the intervillous space where drainage occurs via the endometrial veins. [Taken from Handbook of Placental Pathology, IGAKU-SHOIN Medical Publishers, Inc., 1994].

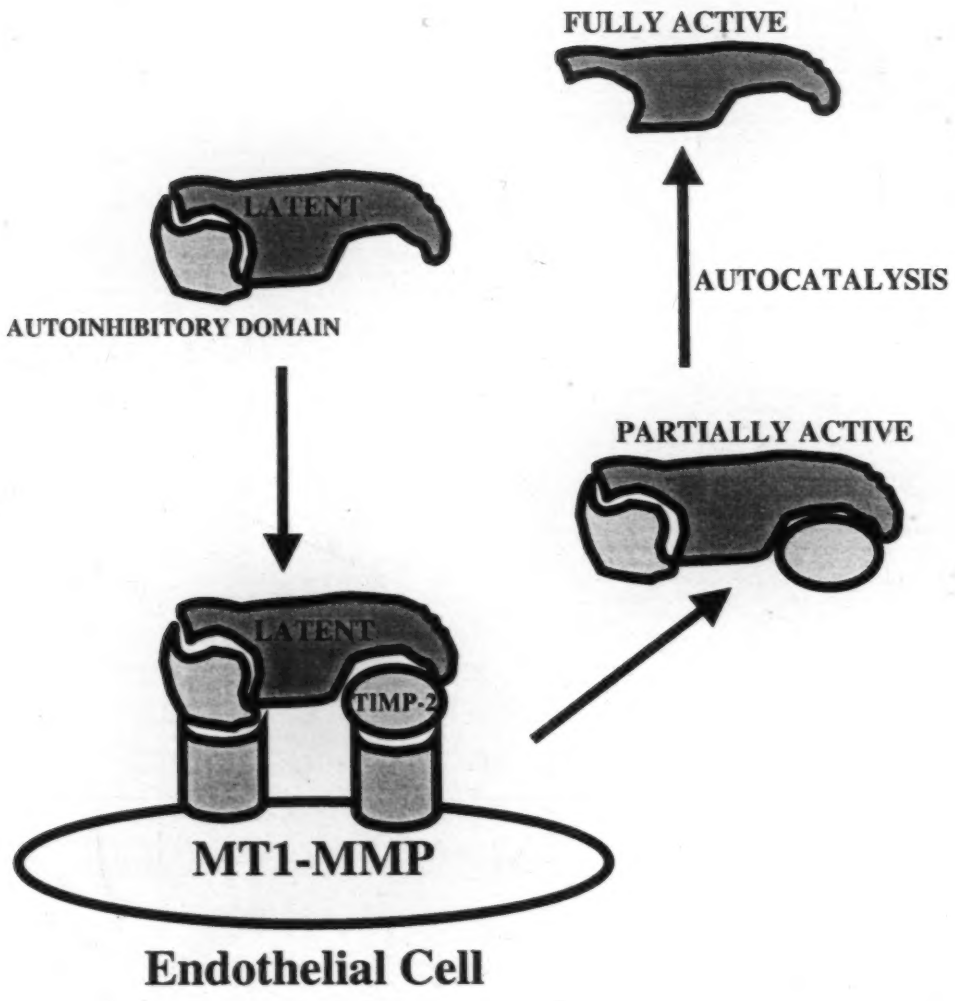

Figure 1.2 Activation of pro-MMP-2 on the surface of endothelial cells.

TIMP-2 binds to the catalytic domain of active MT1-MMP via its N-terminal domain. Pro-MMP-2 interacts with TIMP-2 via its C-terminal domain forming a trimolecular complex. A nearby free MT1-MMP then partially activates pro-MMP-2 by initiating a cleavage in the propeptide domain of pro-MMP-2. The partially active MMP-2 is released and subsequently autocatalysed to the fully active form [adapted from Nguyen et al., 2001].

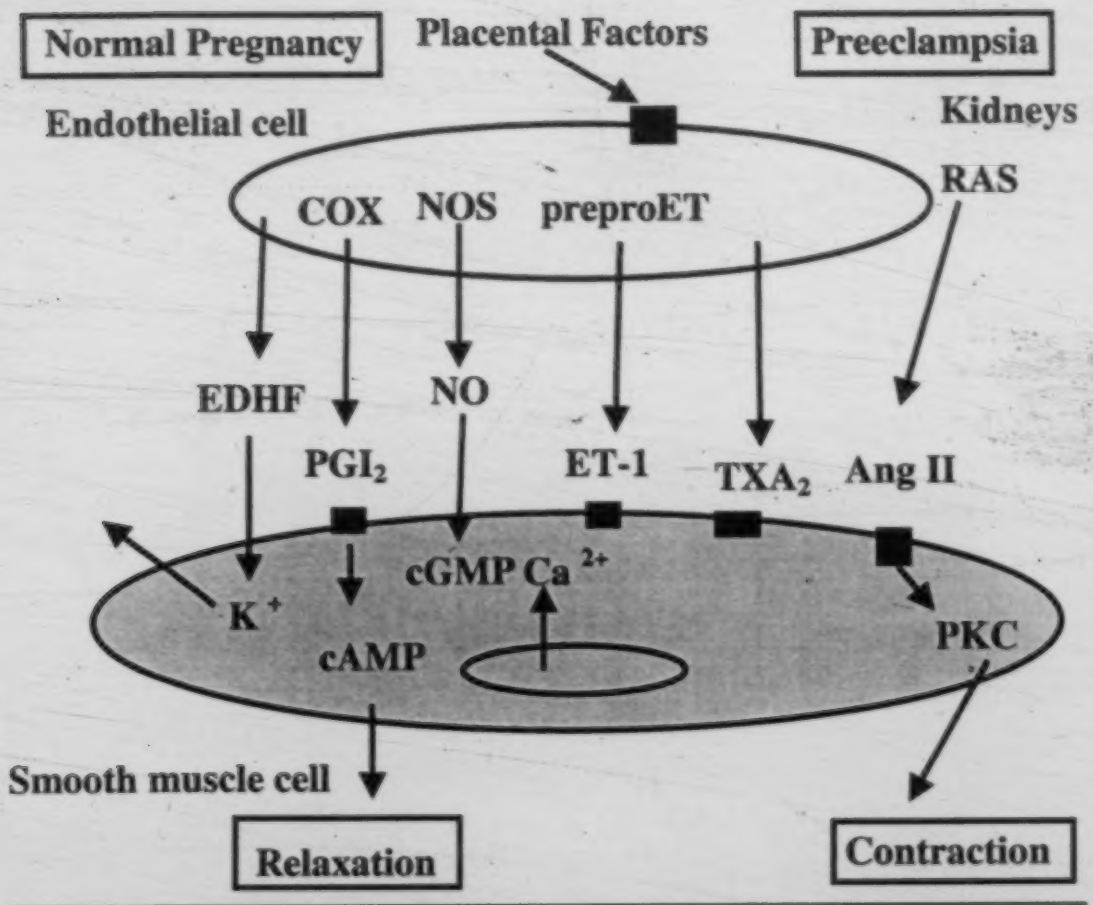

Figure 1.3 Vascular changes during normal pregnancy and preeclampsia.

During normal pregnancy, there is an increase in the activity of endothelial nitric oxide synthase (NOS) and cyclooxygenase (COX) and increased production of nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF). NO increases cGMP and PGI_2 increases cAMP in smooth muscle which decreases intracellular Ca^{2+} and myofilament sensitivity to Ca^{2+}. EDHF open K^+ channels in smooth muscle leading to hyperpolarization. This leads to smooth muscle relaxation and decreased peripheral vascular resistance. In preeclampsia, there is increased release of placental factors that may decrease production of vasodilatory factors. Placental factors may also stimulate the release of vasoconstrictors such as endothelin (ET) and thromboxane (TxA_2) and could activate the renin-angiotensin system (RAS) in the kidney, leading to increased angiotensin II (Ang II). ET-1, TxA_2, and AngII stimulate specific receptors in smooth muscle cells, leading to increased intracellular Ca^{2+}, protein kinase C (PKC) activity, smooth muscle contraction, and increased peripheral vascular resistance. SR = sarcoplasmic reticulum [adapted from Khalil and Granger, 2002].

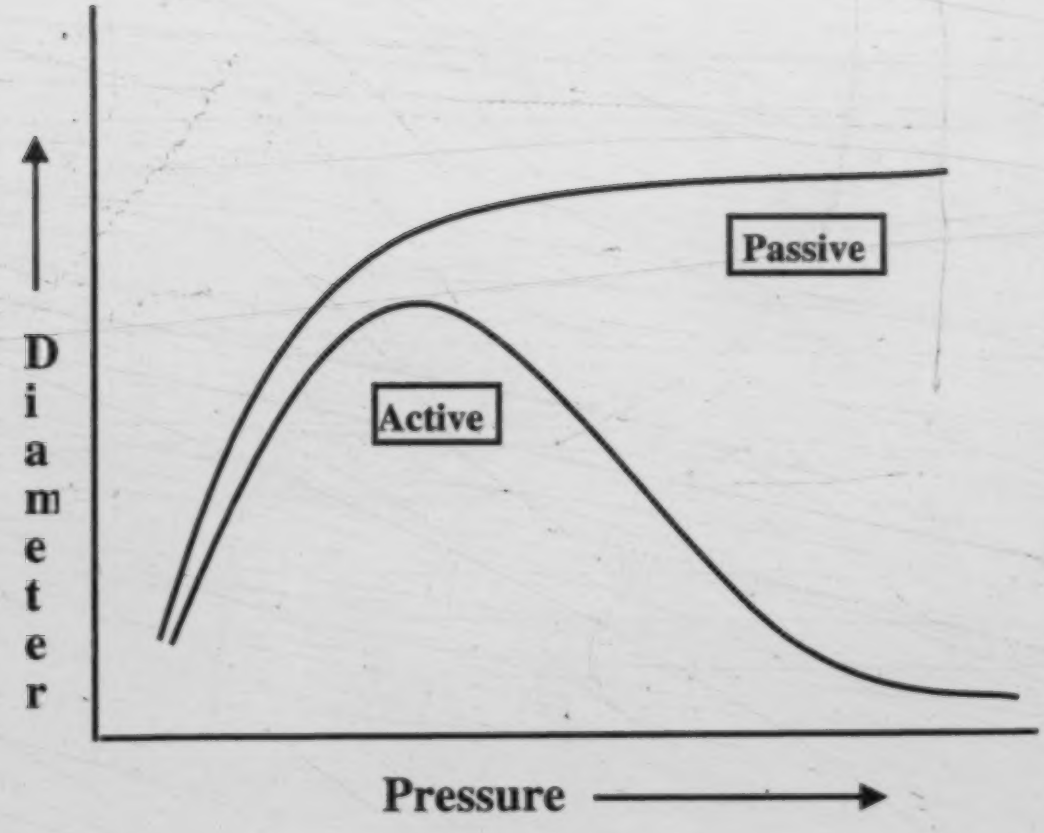

Figure 1.4 An example of myogenic tone in small arteries and arterioles.

The graph illustrates myogenic behaviour with (active) or without (passive) Ca^{2+} in the bathing media. Note that the myogenic response is abolished in the absence of extracellular Ca^{2+}.

CHAPTER 2. MATRIX METALLOPROTEINASE RELEASE FROM PLACENTAL EXPLANTS FROM PREGNANCIES COMPLICATED BY INTRAUTERINE GROWTH RESTRICTION[1].

2.1 INTRODUCTION

Intrauterine growth restriction (IUGR) is a serious complication of pregnancy with perinatal mortality rates up to tenfold greater than normal pregnancies (11). The growth-restricted fetus is at increased risk of perinatal complications such as fetal distress, asphyxia, hypothermia, hypoglycemia, and poor feeding, as well as long-term neurological and developmental disorders (42). Furthermore, IUGR may alter *in utero* programming of the fetus (4) leading to serious health implications in adult life including increased risk of hypertension, heart disease and diabetes (5, 6). Despite extensive investigation, the pathophysiology of IUGR remains unclear.

Placentas from IUGR pregnancies tend to be smaller than those from normal pregnancies (3, 14, 17) and there is a strong correlation between lower placental weights and significantly decreased fetal weights in IUGR (33). Processes that regulate placental growth include placental cell turnover (death and proliferation) and tissue matrix remodeling driven by blood vessel formation (angiogenesis). There is evidence of increased placental apoptosis (programmed cell death) in IUGR (39); however, it is unknown whether tissue matrix remodeling is also altered. Matrix metalloproteinases (MMPs) are critical mediators of placental remodeling (28, 35).

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MMPs are released by a variety of cells including endothelial cells (8) and placental trophoblasts (25), and require cleavage or reconfiguration of the N-terminal propeptide for full activation (26, 41). Their catalytic activity is regulated at multiple levels including transcription, secretion, activation and inhibition by specialized proteins called tissue inhibitors of metalloproteinases (TIMPs) which bind to the MMP active site (26). The resulting steric hindrance prevents interaction of the MMP with components of the extracellular matrix (ECM) (21). The gelatinases MMP-2 and MMP-9 are extensively involved in ECM remodeling through degradation of basement membrane collagens (8) and the balance between MMPs and their inhibitors is likely to determine the extent of this remodeling within the placenta (28, 35). The placental pathologies associated with IUGR pregnancies may reflect a diminished capacity for villous tissue remodeling.

Inadequate delivery of oxygen to the fetus is a characteristic feature of IUGR (19, 36). Oxygen delivery may be insufficient either to, or across the placenta, or both (19). Inappropriate adaptation of the maternal spiral arteries can reduce blood flow to the placenta causing both placental and fetal hypoxia, whereas failure of the fetoplacental circulation to extract oxygen from the intervillous space can lead to placental hyperoxia and fetal hypoxia (19). Interestingly, larger transplacental gradients exist in IUGR pregnancies (32) with higher intervillous and lower umbilical vein oxygen levels (37); thus, placental tissue in IUGR pregnancies may be exposed to both higher and lower oxygen levels than those in normal pregnancies.

With an overall hypothesis that dysregulation of matrix remodeling contributes to impaired placental development, we specifically hypothesized that in pregnancies complicated by IUGR, the release of MMPs from placental explant cultures is decreased and/or there are increased levels of TIMPs compared to explants from normal pregnancies. Because of the potential effects of oxygen gradient differences between

normal and IUGR placentas, we tested our hypothesis under both high (20%) and low (3%) oxygen levels.

2.2 MATERIALS AND METHODS

2.2.1 Tissue Collection and Experimental Protocol

Human term placentas were obtained following normal vaginal deliveries or Caesarean section from 7 normal pregnancies and 7 pregnancies complicated by IUGR [3 of which were also complicated by preeclampsia (PE)] from the delivery unit of St. Mary's Hospital, Manchester, United Kingdom. Samples of placenta were collected from midway between the chorionic and basal plates, from lobes free of visible calcification or tears. Chorionic villi (free floating) were dissected out within 30 minutes of delivery and carefully rinsed in sterile phosphate-buffered saline to remove maternal blood. The villous tissue was cut into pieces of similar weight and four such pieces were cultured in individual Costar Netwell (15-mm diameter, 74 μm mesh; Corning, Corning, NY) supports in 1.5 ml of culture medium (CMRL-1066, 5% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml insulin, 0.1 μg/ml hydrocortisone, and 0.1 μg/ml retinyl acetate; Sigma Chemical Co., Poole, UK.). The tissue was supported on the mesh in the liquid-gas interface. Cultures were maintained at 37°C at 20% O_2 (to simulate high oxygen) and 3% O_2 (to simulate low oxygen). Because explants are chunks of tissue, the interior of the explant may not experience the same oxygen environment as the exterior; therefore, we chose 20% and 3% as two extreme oxygen levels to simulate high and low oxygen in the placenta. Conditions of reduced oxygen were generated using an atmosphere control chamber with purge airlock and forced air incubator (Coy Laboratory Products Inc., Michigan, USA). All dissections

were conducted within these relevant oxygen environments. Culture medium was changed every 24 hours, with pre-equilibration of the media for 24 hours in the chamber prior to adding to the cultures. Supernatants were collected and stored at –80°C for analysis. Supernatants from day 4 of culture were selected for subsequent analyses as they demonstrated peak endocrine function and cellular integrity as indicated by increased human chorionic gonadotropin and decreased lactate dehydrogenase levels, respectively (38). Supernatant samples from one explant from a normal pregnancy cultured at 20% oxygen and one explant from an IUGR pregnancy cultured at 3% oxygen, were discarded as these samples showed evidence of degradation when analyzed by zymography. There were no differences in placental explants complicated by PE and IUGR compared to explants complicated by IUGR alone for our outcome measures (data not shown); therefore, explants from pregnancies complicated by PE and IUGR (n = 3) and IUGR alone (n = 4) were combined to form a single IUGR group in our subsequent analyses. In addition, there were no significant differences in the amounts of protein in the explants cultured during experiments (Normal pregnancy, 20% oxygen: 0.056 ± 0.013 mg; 3% oxygen: 0.082 ± 0.015 mg; IUGR pregnancy, 20% oxygen: 0.061 ± 0.009 mg; 3% oxygen: 0.080 ± 0.009 mg).

IUGR was identified by antenatal ultrasound scans (that demonstrated growth < 10^{th} percentile) and confirmed after delivery by an individualized birthweight ratio (IBR) of below the 10^{th} percentile. The IBR is relative to predicted birthweight and is calculated using independent coefficients for gestational age at delivery, fetal sex, parity, ethnicity, maternal height and pre-pregnant weight (43). The IBR enables a more accurate prediction of pregnancies ending in poor outcome than birthweight for gestational age alone. Preeclampsia was defined as a blood pressure of >140/90 mm Hg on two or more occasions after the twentieth week of pregnancy, in a previously normotensive woman in the presence of significant proteinuria (either >300mg/litre in a 24-hour collection or >2+ on a voided random urine sample in the absence of urinary tract infection).

It is difficult to define criteria for the diagnosis of IUGR (18). The above criteria for IUGR have been used previously (39, 40); nevertheless, sub-classifications of IUGR exist based on Doppler ultrasound findings (i.e. absent or reversed end diastolic flow, preserved end diastolic flow in the umbilical artery) (7). We do not have Doppler ultrasound information for any of the pregnancies in this study.

2.2.2 Gelatin Zymography

Supernatants were collected as described above and protein concentrations were measured (BCA Protein Assay Kit, Pierce, Rockford, IL). The MMP-2 and -9 levels in supernatants were evaluated by zymography according to established methods (12, 15). Zymography detects both the proenzyme and mature forms of MMPs, distinguished on the basis of molecular weight (13) and it quantitatively assesses existing or potential gelatinase activity (20). The electrophoretic process also effectively separates MMPs from possible endogenous inhibitors such as TIMPs (20). It is important to note that the proenzyme form can be activated due to reconfiguration by biological molecules *in vivo* (23, 29, 31) as well as by denaturing conditions in the zymography assay (30, 41).

Briefly, supernatants containing equal protein amounts (7.5 μg) were prepared by dilution in a 6X sample buffer and loaded into lanes of a 7.5% polyacrylamide gel containing SDS copolymerized with 3mg/mL gelatin. Following electrophoresis, the gels were washed in 2.5% Triton X-100 (3 × 20 minutes) and incubated in development buffer (0.15M NaCl, 5mM $CaCl_2$, 0.05% NaN_3, and 50mM Tris-HCl) for 20 hours at 37 °C. The gels were stained in Coomassie blue for 1.5 hours (1.5 g/L Coomassie Brilliant Blue, 25% methanol, 10% acetic acid, 65% water) and destained for 4 hours (4% ethanol, 8%

acetic acid, 88% water). The clear bands indicating gelatinase activity were scanned with a densitometer (Fluor-S MAX MultiImager, Bio-Rad Laboratories, CA) and quantitated using the Quantity One Image Analysis System (Bio-Rad Laboratories, CA).

We detected the inactive (pro) forms of the gelatinases, including the 72 kDa form of MMP-2 and the 92 kDa form of MMP-9 in our zymographies. Signals in culture supernatants were above background levels of gelatinases contained in the medium (data not shown). Release from explants was quantitated by subtracting background levels of MMPs in culture medium from levels in explant culture supernatants. MMP-2 and -9 were identified by electrophoretic mobility in comparison with a standard (human MMP-2/MMP-9 zymography standard, Chemicon International, Tenecula, CA) run concurrently on the gel. HT1080 cells (human fibrosarcoma) secrete MMP-2 and –9 and TIMP-1 and –2 (24, 44); therefore, conditioned medium from these cells was used as a positive control for our outcome measures (5 μg for MMP-2 and –9, 15 μg for TIMP-1). The MMP-2 and -9 signals detected from all samples were within the linear range of our zymography assay (a standard curve was constructed). Because band densities for replicates of the same sample were more variable between different gels than on the same gel, comparisons were always made between samples run on the same gel. MMP-2 and –9 levels in explant culture supernatants were normalized to an internal control (known amount of conditioned medium from HT1080 cells) that was run on all zymograms.

2.2.3 Western Blot Analysis

Cell supernatants were collected as described above and protein concentrations measured (BCA Protein Assay Kit, Pierce, Rockford, IL). In order to detect TIMP-2, we concentrated our supernatant samples 5-fold (Millipore Ultra-free protein concentrators,

molecular weight cut off of 5000 kDa) and then measured protein concentrations. Supernatants containing equal protein amounts (60 μg) were reduced and denatured (by boiling for 5 minutes) in a 5X sample buffer (1 mL 0.5M Tris-HCl pH 6.8, 1 mL β-mercaptoethanol, 4 mL glycerol, 2 mL 20% SDS, 4 mg bromophenol blue), then loaded on an 8% gel run at 60 V for 30 minutes and 140 V for an additional 40 – 60 minutes in running buffer. Gels were stained with Coomassie blue to verify the protein load. Following electrophoresis, the gel, nitrocellulose membranes and blotting filters were equilibrated for 10 minutes in transfer buffer (9.0g Tris, 43.2g glycine, 600 mL methanol, water added to 3 L). Proteins were then transferred electrophoretically using a transfer apparatus (Bio-Rad Laboratories, CA) overnight at 4 °C at 20V, followed by 100V for 40 minutes. The nitrocellulose membranes were washed in PBS with 0.05% Tween (TPBS) (3 × 10 minutes) and blocked for 3 hours at room temperature in 5% milk powder diluted in TPBS. The membranes were incubated in monoclonal primary antibodies directed at TIMP-1 (1:100, Calbiochem, San Diego, CA) and TIMP-2 (1:100, Calbiochem, San Diego, CA). The primary antibodies were detected with horse radish peroxidase-conjugated secondary antibodies using an ECL Chemiluminescence kit (Amersham Pharmacia Biotech, England, UK). Protein bands were compared to a molecular weight marker (Broad-range unstained marker, Bio-Rad Laboratories, CA) that was run concurrently on the gel. TIMP-1 levels in explant culture supernatants were normalized to an internal control as described for MMP-2 and –9 levels.

2.2.4 Statistics

A Student's t-test was used to compare between two groups. Significance was considered when $p < 0.05$.

2.3 RESULTS

Patient demographics are summarized in **Table 2.1**. Women with PE and IUGR (n = 3) had significantly elevated blood pressure ($p < 0.001$) and shorter gestation ($p < 0.05$) compared to normal pregnant women (n = 7) and women with IUGR (n = 4). Women with PE and IUGR and IUGR alone gave birth to significantly smaller babies compared to women with normal pregnancies ($p < 0.001$).

2.3.1 Comparison between normal and IUGR explants for release of MMP and TIMP at different oxygen levels

Because there was much less variability in sample detection on the same than different gels for zymography and Western blot analysis (see Materials and Methods), we carried out separate zymographic analyses comparing release of MMP-2 from normal and IUGR explants at 20% oxygen (**Figure 2.1A**) and 3% oxygen (**Figure 2.1B**). MMP-9 release was compared in the same fashion from the same two gels at 20% oxygen (**Figure 2.1C**) and 3% oxygen (**Figure 2.1D**). TIMP-1 release from normal and IUGR explants was compared on the same Western blot at 20% oxygen (**Figure 2.2A**) and 3% oxygen (**Figure 2.2B**).

We detected the pro-forms of the gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa) in the supernatants of placental explant cultures. Explants from pregnancies complicated by IUGR released significantly less MMP-2 compared to explants from normal pregnancies (~ 21%; $p < 0.05$) when cultured at 20% oxygen (**Figure 2.1A**). However, when exposed to low oxygen (3%), MMP-2 release was the same in explants from normal and IUGR pregnancies (**Figure 2.1B**). There was a trend indicating reduced MMP-9 release in

explants from IUGR compared to normal pregnancies when cultured at 20% oxygen (~ 42%; $p = 0.07$; **Figure 2.1C**). When cultured at 3% oxygen there was even less difference in MMP-9 release from explants from normal and IUGR pregnancies (**Figure 2.1D**).

We detected TIMP-1 (29 kDa), but not TIMP-2, in supernatants from placental explant cultures. TIMP-1 release was significantly reduced in explants from IUGR compared to normal pregnancies when cultured at 20% oxygen (by ~ 66%; $p < 0.01$; **Figure 2.2A**). When cultured at 3% oxygen, this difference was less pronounced; however, there was a trend indicating decreased TIMP-1 release from explants from IUGR pregnancies (~ 50%; $p < 0.067$; **Figure 2.2B**).

2.3.2 The effect of oxygen level on MMP and TIMP release from normal and IUGR explants

We next compared MMP-2 release from normal (**Figure 2.3A**) and IUGR (**Figure 2.3B**) explant cultures carried out at high (20%) and low (3%) oxygen levels, each figure summarizing a single zymography gel. The relative release of MMP-9 from normal (**Figure 2.3C**) and IUGR (**Figure 2.3D**) explants in 20% and 3% explant cultures was evaluated in the same two gels. The relative release of TIMP-1 in 20% and 3% explant cultures is depicted in the summaries of the Western blots for explants from normal (**Figure 2.4A**) and IUGR (**Figure 2.4B**) placentas.

MMP-2 release was significantly reduced at 3% compared to 20% oxygen in placental explants from normal ($54 \pm 5\%$; $p < 0.001$) and IUGR ($22 \pm 11\%$; $p < 0.05$) pregnancies (**Figure 2.3A and B,** respectively). There were no significant effects of varying oxygen on MMP-9 release in explants from normal (**Figure 2.3C**) and IUGR (**Figure 2.3D**)

pregnancies, although its expression became more variable at 3% oxygen. TIMP-1 release was significantly reduced at 3% oxygen compared to 20% oxygen in explants from normal (83 ± 3%; $p < 0.005$) and IUGR (59 ± 13%; $p < 0.05$) pregnancies (**Figure 2.4A and B,** respectively).

2.4 DISCUSSION

In our studies, placental explants from pregnancies complicated by IUGR demonstrated significantly decreased MMP-2 and TIMP-1 release and a trend toward reduced MMP-9 release at high oxygen. This suggests that the placental abnormalities observed in some IUGR pregnancies may be due in part to decreased levels of ECM-remodeling enzymes under conditions of placental hyperoxia. IUGR pregnancies are associated with increased uterine vein PO_2, but decreased umbilical vein PO_2 and uterine oxygen extraction (37), suggesting intervillous hyperoxia. Furthermore, levels of vascular endothelial growth factor (VEGF) (37) and placenta growth factor (PlGF) (16) are decreased and increased, respectively, in IUGR placentas, again suggesting placental hyperoxia. Interestingly, the increase in MMP-2 release in high oxygen compared to low oxygen cultures was significantly smaller in IUGR than normal explants (22 ± 11% vs. 54 ± 5%, respectively) and all of the difference can be ascribed to lower production by IUGR explants at high oxygen. Collectively, these observations suggest that the failure of IUGR placentas to grow properly reflects a pathologic interaction between higher than normal oxygen levels in the intervillous space and an inherent inability to accommodate higher oxygen.

The placentas from IUGR pregnancies tend to be smaller than those from normal pregnancies (3, 14, 17) and this may be due to an imbalance of processes that regulate placental growth. Smith et al. (40) reported no differences in cellular proliferation but increased apoptosis (39) in placentas from IUGR compared to normal pregnancies. In this

study, we provide evidence of decreased levels of matrix-degrading enzymes (as indicators of tissue remodeling) in IUGR placentas at high oxygen. These results suggest that impaired tissue remodeling may also contribute to placental maldevelopment. Additional factors, particularly those that affect MMP release, may also contribute to placental maldevelopment in IUGR. For example, VEGF stimulates MMP release from endothelial cells (27) and VEGF mRNA in placentas (10) and protein in syncytiotrophoblasts and villous endothelial cells (22, 37), is reduced in placentas complicated by PE and IUGR, suggesting higher than normal placental oxygen levels in these pregnancies. Because of its critical role in angiogenesis (9), these results collectively suggest that reduced VEGF expression due to high oxygen may be responsible, in part, for decreased levels of ECM-remodeling enzymes and impaired placental vascular development in these conditions.

We were surprised to see a significant decrease in MMP-2 release in response to 3% oxygen in explants from both normal and IUGR pregnancies. Our previous studies in cultured human umbilical vein endothelial cells have shown higher MMP-2 release at 2% compared to 20% oxygen conditions. Typically, angiogenesis is increased in response to hypoxia through increased expression of VEGF (1, 2), which may influence MMP secretion (27). Placental explants are complex structures composed of endothelial cells, macrophages, fibroblasts, and trophoblasts in distinct anatomical compartments (7), and all of these cell types can secrete MMPs (8, 25, 34); consequently, the lower levels of MMP-2 at 3% compared to 20% oxygen may reflect highly integrated or separated cellular processes rather than simply intravillous angiogenesis. For example, placental endothelial cells may perform differently in the context of intact placental tissue, or MMP release from explants may not reflect intravillous levels.

In conclusion, we measured release of MMPs and their inhibitors from placental explants under conditions simulating high and low oxygen. We demonstrated reduced release of

MMP-2, MMP-9 and TIMP-1 from explants from IUGR pregnancies at high oxygen, suggesting that the placental maldevelopment in IUGR pregnancies is more likely to occur as a result of hyperoxic conditions. We also observed that IUGR explants are less responsive to changes in oxygen tension, reflected in our study, by MMP-2 release. We suggest that placentas from IUGR pregnancies have a diminished capacity to respond to high oxygen, which may exist in the broad transplacental gradient associated with an IUGR pregnancy.

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Characteristic	Normal Pregnant (n = 7)	Preeclampsia + IUGR (n = 3)	IUGR (n = 4)
Maternal Age (years)	25.1 ± 3.4	27.3 ± 3.3	25 ± 4.9
Term BP (mm Hg)	118/72 ± 5/4	167/110 ± 6/6 *	116/70 ± 4/0
Gestation (weeks)	39.7 ± 0.8	31.7 ± 3.8 *	37.8 ± 0.3
Infant birth weight (g)	3597 ± 274	1303 ± 304 **	2245 ± 270 **

Table 2.1 Patient demographics of women at St. Mary's Hospital, Manchester, United Kingdom, from whom placentas were obtained.

BP = blood pressure; *P < 0.05 vs. normal pregnancies and pregnancies complicated by IUGR; **P < 0.05 vs. normal pregnancies.

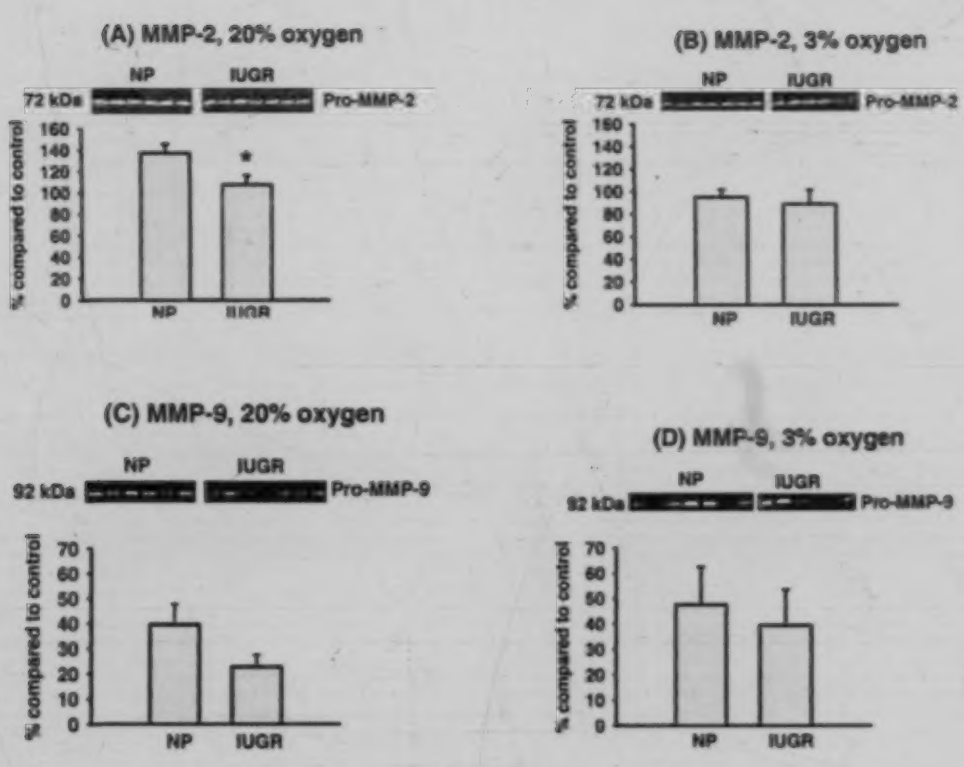

Figure 2.1 Comparison between explants from normal and IUGR pregnancies for release of MMP-2 and MMP-9 at different oxygen levels.

Zymographic analysis of **MMP-2 release** from placental explants from normal pregnant (NP) and IUGR pregnancies cultured for 24 hours at high (20%) oxygen (n = 6, NP; n = 7, IUGR; **A**) and low (3%) oxygen (n = 7, NP; n = 6, IUGR; **B**). Also shown is **MMP-9 release** from placental explants from NP and IUGR pregnancies cultured for 24 hours at high (20%) oxygen (n = 6, NP; n = 7, IUGR; **C**) and low (3%) oxygen (n = 7, NP; n = 6, IUGR; **D**). The upper panels show the zymograms (7.5 μg of protein per lane) of MMP-2 (**A** and **B**) and MMP-9 (**C** and **D**) release. The lower panels show summary data as percent compared to the control (5 μg of conditioned medium from HT1080 cells). The data are presented as means ± SEM of the percent compared to the control. * $p < 0.05$ at 20% oxygen for MMP-2. Although there was a trend indicating decreased MMP-9 release in explants from IUGR pregnancies compared to those from normal pregnancies at 20% oxygen, it was not statistically significant ($p = 0.07$).

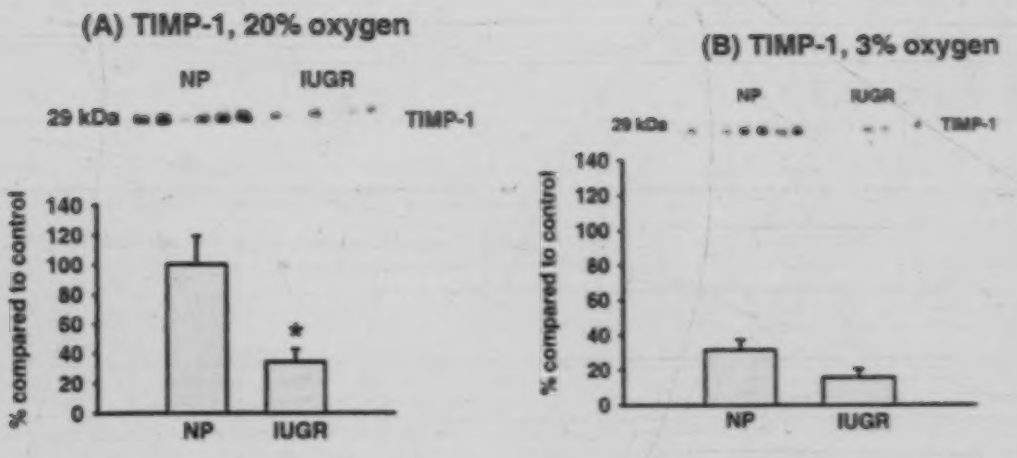

Figure 2.2 Comparison between explants from normal and IUGR pregnancies for release of TIMP-1 at different oxygen levels.

Western blot analysis of **TIMP-1 release** from placental explants from normal pregnant (NP) and IUGR pregnancies at high (20%) oxygen (n = 6, NP; n = 7, IUGR; **A**) and low (3%) oxygen (n = 7, NP; n = 6, IUGR; **B**). The upper panels show the Western blot (60 μg of protein per lane) of TIMP-1 release. The lower panels show the summary data as percent compared to control (15 μg of conditioned medium from HT1080 cells). The data are presented as means ± SEM of the percent compared to the control. * $p < 0.01$ at 20% oxygen.

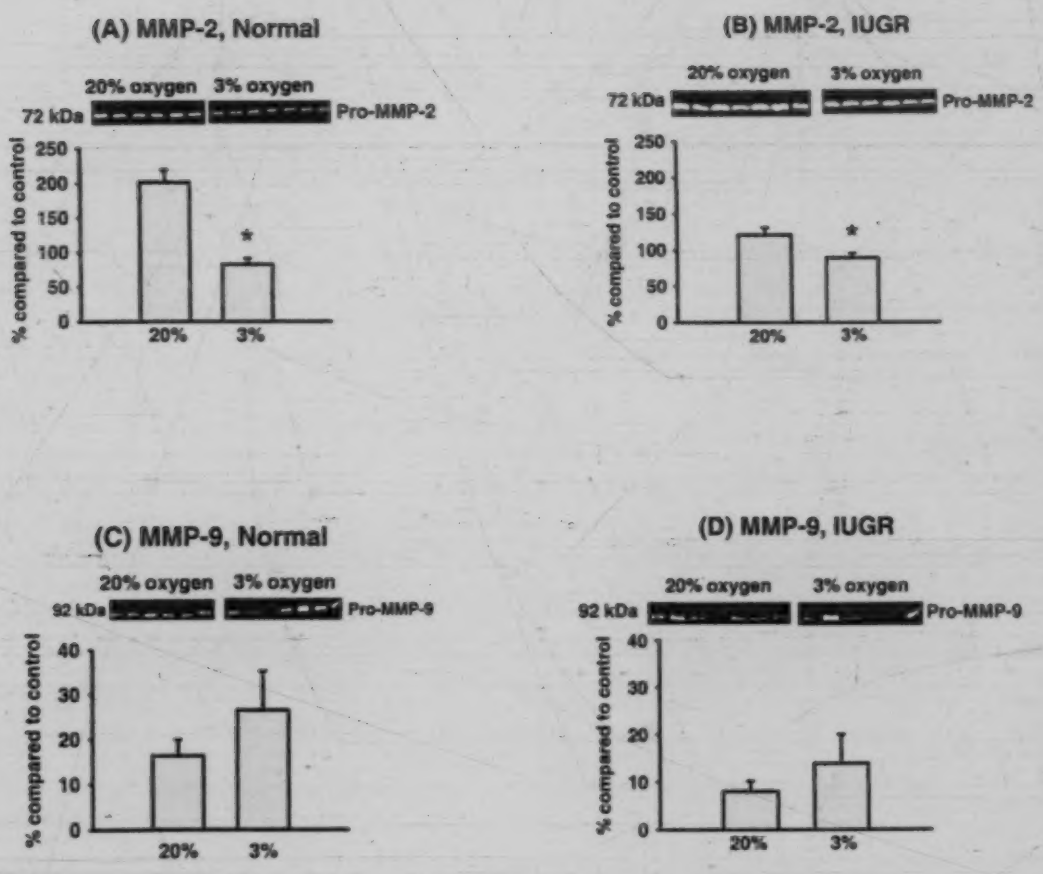

Figure 2.3 The effect of oxygen level on MMP-2 and MMP-9 release from explants from normal and IUGR pregnancies.

Zymographic analysis of **MMP-2 release** from placental explants from normal (n = 6, 20%; n = 7, 3%; **A**) and IUGR (n = 7, 20%; n = 6, 3%; **B**) pregnancies in response to high (20%) and low (3%) oxygen levels. Also shown is **MMP-9 release** from placental explants from normal (n = 6, 20%; n = 7, 3%; **C**) and IUGR (n = 7, 20%; n = 6, 3%; **D**) pregnancies in response to high (20%) and low (3%) oxygen levels. The upper panels show the zymogram (7.5 μg of protein per lane) of MMP-2 (**A** and **B**) and MMP-9 (**C** and **D**) release. The lower panels show summary data as percent compared to the control (5 μg of conditioned medium from HT1080 cells). The data are presented as means ± SEM of the percent compared to the control. * $p < 0.001$ for explants from normal pregnancies and * $p < 0.05$ for explants from IUGR pregnancies for MMP-2 release.

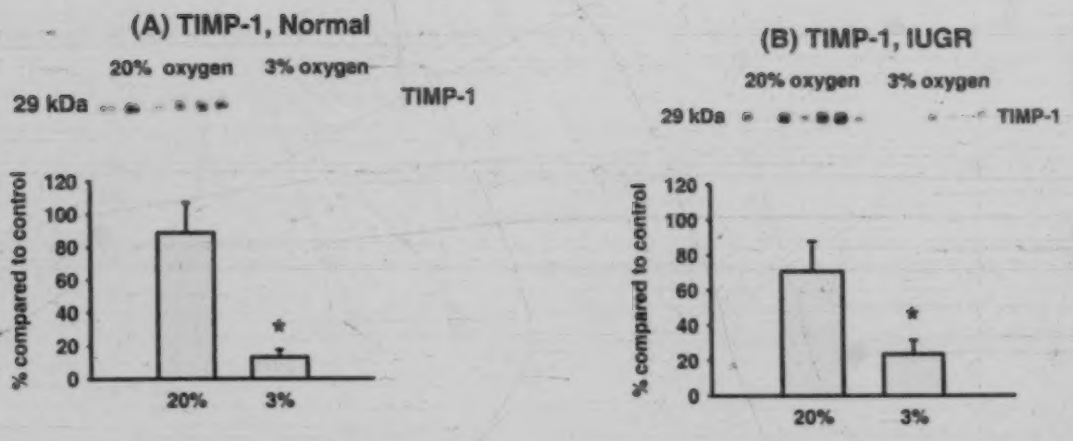

Figure 2.4 The effect of oxygen level on MMP-2 and MMP-9 release from explants from normal and IUGR pregnancies.

Western blot analysis of **TIMP-1 release** from placental explants from normal (n = 6, 20%; n = 7, 3%; **A**) and IUGR (n = 7, 20%; n = 6, 3%; **B**) pregnancies in response to high (20%) and low (3%) oxygen levels. The upper panels show the Western blot (60 µg of protein per lane) of TIMP-1 release. The lower panels show the summary data as percent compared to control (15 µg of conditioned medium from HT1080 cells). The data are presented as means ± SEM of the percent compared to the control. * $p < 0.005$ for explants from normal pregnancies and * $p < 0.05$ for explants from IUGR pregnancies.

CHAPTER 3. THE EFFECTS OF PREECLAMPSIA AND OXYGEN ENVIRONMENT ON ENDOTHELIAL RELEASE OF MATRIX METALLOPROTEINASE-2[2].

3.1 INTRODUCTION

Despite decades of extensive investigation, the etiology and pathophysiology of preeclampsia remain poorly understood (14, 29, 43, 44). Traditionally, preeclampsia has been classified as a hypertensive disease of pregnancy; nevertheless, it affects virtually all organ systems, with hypertension representing only one manifestation (14, 29, 43, 44).

There is evidence that endothelial cell activation/alteration is central to the pathophysiology of preeclampsia (20, 25, 43, 45), an appealing hypothesis because the vascular endothelium is a ubiquitous organ and may account for the multi-system effects of the disease. Biochemical evidence supports the existence of agents that are released by activated endothelial cells, including von Willebrand factor (44), endothelin (51), and the cellular epitope of fibronectin (9, 50). Dysfunction of the endothelium may lead to a state of generalized vasoconstriction, platelet activation and organ hypoperfusion, commonly observed in preeclampsia. The etiology that causes endothelial dysfunction remains unclear.

One recently described mediator of vascular reactivity is matrix metalloproteinase (MMP)-2, which is found at significantly elevated levels in the plasma of preeclamptic

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women (35). MMP-2 belongs to a family of extracellular matrix degrading enzymes that are secreted as partially active zymogens and require proteolytic cleavage or reconfiguration (33, 48) of the N-terminal propeptide for full activation. Specific to our study, MMP-2 is a 72 kDa gelatinase that is produced by many cell types including endothelial cells and that degrades basement membrane collagens. In addition to the known effects of MMP-2 on vascular remodeling, we previously demonstrated that it can mediate vascular reactivity by cleaving big endothelin to yield a potent vasoconstrictor (12) as well as reduce vasorelaxation through cleavage of calcitonin gene-related peptide (13).

Oxygen is known to be a regulator of MMP activity either directly via oxygen sensitive transcription factors on MMP genes (22), or indirectly through cytokines and growth factors (32, 41). We hypothesized that human umbilical vein endothelial cells (HUVECs) from preeclamptic pregnancies would show significantly enhanced MMP-2 release compared to cells from normal, uncomplicated pregnancies and that this phenomenon may be mediated by an oxygen-sensitive mechanism.

3.2 MATERIALS AND METHODS

3.2.1 HUVEC Isolation and Culture

Umbilical cords were obtained after delivery from women with uncomplicated pregnancies (n = 10) and women with preeclampsia (n = 4). Preeclampsia was defined using the criteria of hypertension and proteinuria developing after the 20th week of gestation. Hypertension was defined as an absolute blood pressure >140/90 mm Hg on two occasions at least 6 hours apart. Proteinuria was defined as >500 mg/24 hours

collection or 2+ on a urine Dipstix test. The women with uncomplicated pregnancies were normotensive throughout gestation and had no proteinuria. No subject was known to have a history of chronic hypertension, liver, renal, or metabolic disease.

HUVECs were isolated by collagenase digestion and cultured on gelatin-coated flasks in M199 media (Gibco) supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine, 2% penicillin-streptomycin, and 1% endothelial cell growth supplement (VWR Canlab). Their endothelial nature was confirmed by anti-human Von Willebrand factor staining (Sigma). Cells were maintained at 37 °C, 5% CO_2 and 20% oxygen until experimentation.

3.2.2 Preparation of HUVEC-Conditioned Medium

HUVECs (passages 2 and 3) from uncomplicated (n = 4) and preeclamptic (n = 4) pregnancies were propagated on gelatin-coated 6-well dishes at a concentration of 500,000 cells/well. Upon reaching confluence, medium was removed and the cells were incubated for 12 hours at 20% oxygen in 1 mL of Q-media (Gibco, phenol-red free M199 media, 1% FBS, 2% penicillin-streptomycin, 1% L-glutamine). The use of Q-media in experiments reduced endogenous metalloproteinases found in serum, enabling accurate detection of enzymes released from cells. The 12 hour time point allowed optimal detection and quantification of MMP-2 via zymography and densitometry, compared to other time points tested (2 and 6 hours). The media collected from the cultures was centrifuged at 12,000 rpm for 10 minutes to remove cellular debris and the supernatant was aliquoted into Eppendorf tubes and stored at –20 °C. Each aliquot was used only once.

In a separate series of experiments, HUVECs from uncomplicated pregnancies (n = 6, passages 2 and 3) were propagated on gelatin-coated 6-well dishes as described above. However, upon addition of 1 mL of Q-media, the cells were incubated at <0.5%, 2% and 20% oxygen for 12 hours. A <0.5% oxygen environment was obtained using a chamber (Billups-Rothenberg, DeMar, CA) within a CO_2 water jacketed incubator (Forma Scientific) that was purged with a gaseous mixture of 95% N_2 and 5% CO_2 for 15 minutes and then sealed. A 2% oxygen environment was obtained using an oxygen-regulated incubator (Forma Scientific CO_2 water jacketed incubator, Series II, Forma, Marietta, OH). A 20% oxygen environment was obtained using a standard laboratory incubator (NAPCO 5400). Supernatants were collected as described above. Sample protein content was measured with the BCA Protein Assay Kit (Pierce, Rockford, IL) using bovine albumin as a standard. MMP-2 activity was detected using zymography as described below. Western blot analysis for TIMP-1 and -2 and lactate dehydrogenase (LDH) assays to measure cell lysis, were also conducted as described below.

3.2.3 Gelatin Zymography

Zymography detects both the proenzyme (72 kDa) and mature (cleaved, 62 kDa) forms of MMPs, distinguished on the basis of molecular weight (19) and it quantitatively assesses existing or potential gelatinase activity (26). The electrophoretic process also effectively separates MMPs from possible endogenous inhibitors such as TIMPs (26). It is important to note that the proenzyme form can be activated due to reconfiguration by biological molecules *in vivo* (30, 37, 40) as well as by denaturing conditions in the zymography assay (39, 48).

The MMP-2 levels in supernatants were evaluated by zymography according to established methods (18, 21), with some modifications. Briefly, samples containing equal

protein amounts (5μg-7.5μg) were prepared by dilution in a 6X sample buffer and loaded without prior boiling into lanes of a 7.5% polyacrylamide gel containing SDS copolymerized with 3mg/mL gelatin. Following electrophoresis, the gels were washed in 2.5% Triton X-100 (3 × 20 minutes) and incubated in development buffer (0.15M NaCl, 5mM $CaCl_2$, 0.05% NaN_3, and 50mM Tris-HCl) for 20 hours at 37 °C. The gels were stained in Coomassie blue for 1.5 hours (25% methanol, 10% acetic acid, 65% water) and destained for 4 hours (4% ethanol, 8% acetic acid, 88% water). The clear bands indicating gelatinase activity were scanned with a densitometer (Fluor-S MAX MultiImager, Bio-Rad Laboratories, CA) and quantitated using the Quantity One Image Analysis System (Bio-Rad Laboratories, CA).

We detected primarily the 72 kDa form of MMP-2 in our zymographies and its signal in cell supernatants was above background levels of MMP-2 (by ~ 400%) contained in Q-media. MMP-2 was identified by electrophoretic mobility in comparison with an MMP-2 standard (human MMP-2/MMP-9 zymography standard, Chemicon International, Tenecula, CA) run concurrently on the gel. The MMP-2 signal detected from all samples was within the linear range of our zymography assay and there was sufficient gelatin within the gels to avoid substrate exhaustion (data not shown). The samples from each experiment examining the effect of oxygen on MMP-2 release from normal cells, were run on a single gel and the corresponding densitometric units from each sample were normalized to a control (MMP-2 release at 20% oxygen), thus enabling comparisons between repetitions of the experiment. The samples from experiments comparing MMP-2 release from normal and preeclamptic cells were run on a single gel.

3.2.4 Western Blot Analysis

Cell supernatants were collected as described above, concentrated with protein concentrators (Millipore, Ultra-free protein concentrator, molecular weight cut off 5000 kDa), and protein concentrations were measured (BCA Protein Assay Kit, Pierce, Rockford, IL). Samples containing equal protein amounts (60 μg) were reduced using a 5X sample buffer (1 mL 0.5M Tris-HCl pH 6.8, 1 mL β-mercaptoethanol, 4 mL glycerol, 2 mL 20% SDS, 4 mg bromophenol blue) and denatured by boiling for 5 minutes prior to electrophoresis on an 8% gel at 60 V for 30 minutes and 140 V for an additional 40 - 60 minutes in running buffer. Gels were stained with Coomassie blue to verify the protein load. Following electrophoresis, the gel, nitrocellulose membranes and blotting filters were equilibrated for 10 minutes in transfer buffer (9.0g Tris, 43.2g glycine, 600 mL methanol, water added to 3 L). Proteins were then transferred electrophoretically using a transfer apparatus (Bio-Rad Laboratories, CA) overnight at 4 °C at 20V, followed by 100V for 40 minutes. The nitrocellulose membranes were washed in PBS with 0.05% Tween (TPBS) (3 × 10 minutes) and blocked for 3 hours at room temperature in 5% milk powder diluted in TPBS. The membranes were incubated in monoclonal primary antibodies directed at TIMP-1 (1:100, Calbiochem, San Diego, CA) and TIMP-2 (1:100, Calbiochem, San Diego, CA). The primary antibodies were detected using horse radish peroxidase conjugated secondary antibodies. Signals were detected using an ECL Chemiluminescence kit (Amersham Pharmacia Biotech, England, UK).

3.2.5 LDH Assays for Cell Lysis

A commercially available kit (Sigma Diagnostics) was used to measure LDH levels as an indication of cell lysis (according to protocol) in supernatants of cells from preeclamptic and uncomplicated pregnancies. Absorbance was measured on a platereader (Molecular Devices, UV Max, Kinetic Microplate Reader, Menlo Park, CA) at 462 nm. The assay measures LDH activity in Berger Broida (B-B) units per mL, with a sensitivity range

from 0 B-B units/mL (0 LDH activity) to 2000 B-B units/mL (maximum LDH activity). A sample that contains all lysed cells has an LDH activity of ~ 1,600 B-B Units/mL. LDH activity was approximately 0-100 B-B units/mL in all supernatant samples, suggesting low levels of cell lysis.

3.2.6 Statistics

For cell culture experiments results are presented as means ± SEM of at least four separate experiments. The Student's t-test or a one-way analysis of variance was used to test for differences in the data. When significant differences were found, the Tukey multiple-comparison test was used (Jandel Sigma Stat statistical software). Statistical significance was considered when $p < 0.05$.

3.3 RESULTS

Patient demographics are summarized in **Table 3.1**. Women with preeclampsia (n = 4) had significantly elevated blood pressure and proteinuria compared to normal pregnant women (n = 10). There were no differences in the types of anesthetics (either epidural or spinal) administered during labour in either group. One preeclamptic patient was treated with magnesium sulphate and another received labetalol, an anti-hypertensive medication.

We first investigated MMP-2 release from preeclamptic (n = 4) and normal (n = 4) endothelial cells by gelatin zymography (**Figure 3.1**). Cells cultured for 12 hours in Q-media secreted primarily the pro form of MMP-2 (72 kDa). Zymographic analysis

indicates that MMP-2 release is ~ 280% higher in cells from preeclamptic compared to uncomplicated pregnancies ($p < 0.05$).

TIMP-1 and -2 release were also investigated in these samples by Western blot analysis (**Figure 3.2**). TIMP-1, which primarily binds the gelatinase MMP-9 but also binds to MMP-2, is detected in culture supernatants as an immunoreactive band with a molecular weight of 28 kDa. As illustrated in **Figure 3.2A**, TIMP-1 release is ~ 90% greater in cells from preeclamptic compared to uncomplicated pregnancies ($p < 0.001$). TIMP-2, which has high affinity for MMP-2, is detected in culture supernatants as an immunoreactive band with a molecular weight of 21 kDa. **Figure 3.2B** shows that TIMP-2 release is ~ 330% greater in cells from preeclamptic compared to uncomplicated pregnancies ($p = 0.010$).

The effect of varying oxygen levels (<0.5%, 2%, 20%) on MMP-2 release from cells from uncomplicated pregnancies (n = 6) was also investigated by gelatin zymography (**Figure 3.3**). Data are normalized to MMP-2 release at 20% oxygen. Normal cells demonstrate a biphasic response to varying oxygen levels, as incubation of the cells at 2% oxygen significantly enhances MMP-2 release compared to <0.5% and 20% oxygen ($p < 0.05$). MMP-2 release at 2% oxygen is ~ 60% higher compared to that observed at 20% oxygen. MMP-2 release at <0.5% and 20% oxygen is not significantly different.

TIMP-1 and -2 release were also investigated in these samples and the results are shown in **Figure 3.4**. The figures illustrate that TIMP-1 (**Figure 3.4A**) and -2 (**Figure 3.4B**) release from normal cells does not significantly change with varying oxygen levels. Data are normalized to TIMP-1 (**Figure 3.4A**) and -2 (**Figure 3.4B**) release at 20% oxygen.

3.4 DISCUSSION

Here we report the novel observation that HUVECs from preeclamptic pregnancies show enhanced MMP-2, TIMP-1 and -2 release compared to cells from uncomplicated pregnancies, a possible indication of endothelial dysfunction in the fetal compartment. In support of this concept, it was recently reported that HUVECs derived from preeclamptic pregnancies have altered cation membrane permeability and activity of the endothelial nitric oxide synthase pathway (49). Similarly, Wang et al. (54) reported increased endothelial cell permeability in preeclamptic HUVECs, possibly reflecting disorganized and diminished expression of endothelial cell junctional proteins. A number of other investigators have reported marked morphological abnormalities (8, 10, 16) and alterations of passive and active mechanical properties (4) in umbilical vessels of preeclamptic women compared to women with uncomplicated pregnancies. The cells from the umbilical vessels also demonstrate abnormal production and release of factors such as fibronectin (10) and nitric oxide and prostacyclin (42). Collectively, the data imply that the fetal-derived cells of the umbilical cords from preeclamptic pregnancies demonstrate altered endothelial function, observations that are reaffirmed by our data.

We previously demonstrated that MMP-2 alters vascular function through cleavage of vasoactive peptides, leading to increased vasoconstriction and reduced vasodilation (12, 13). Furthermore, we also reported that MMP-2 levels are elevated in the plasma of preeclamptic women (35). In this study we provide evidence that endothelial cells from umbilical vessels from preeclamptic pregnancies exhibit significantly greater MMP-2, TIMP-1 and -2 release compared to cells from vessels from uncomplicated pregnancies, and propose that variations in oxygen tension contribute to this phenomenon. Indeed, cells from uncomplicated pregnancies show significantly enhanced MMP-2 release at 2% oxygen compared to <0.5% and 20% oxygen; however, the magnitude of the enhanced release is small when compared to the differences in cells from preeclamptic and normal

pregnancies (~ 60% compared to ~ 280% increase). Similarly, cells from preeclamptic pregnancies show enhanced TIMP-1 and -2 release compared to cells from uncomplicated pregnancies; however, TIMP-1 and -2 release from normal cells did not change with varying oxygen levels. Based on our data, it is unlikely that oxygen is a key mediator of the enhanced MMP-2, TIMP-1 and -2 release observed in the preeclamptic endothelial cells, supporting a previous suggestion that there may be an inherent functional abnormality in cells of preeclamptic origin (15).

We are not the first investigators to show oxygen regulation of MMP-2 release; however, our data are novel in regard to demonstrating a biphasic response to varying oxygen levels. Ben-Yosef et al. (3) showed that exposure of human macrovascular endothelial cells to long-term hypoxia (48 hours, PO_2 = 23 ± 4 mm Hg) led to enhanced MMP-2 mRNA and enzyme secretion. Orphanides et al. (38) reported reduced MMP-2 expression by proximal tubular cells from the kidney upon exposure to hypoxic conditions, and similarly, Eddy (11) showed that ischemia in the kidneys led to a decrease in MMPs. In response to hyperoxia, Pardo et al. (41) showed significantly increased levels of MMP-2 and MMP-9 in type II alveolar cells obtained from rats. Investigators have also reported no change in MMP-2 expression by normal and malignant cells under hypoxic conditions (6, 22, 27). The various observations suggest that the activity and role of MMPs in response to varying oxygen levels may depend on the tissue and cell type, as well as the duration and direction of the change in oxygen environment compared to the physiological level.

The mechanisms of oxygen-mediated MMP-2 release are likely complex and may be due to a variety of factors. The metalloproteinase genes are stimulated by a variety of biologically active agents, including growth factors, oncogenes and cytokines (32, 41). Sehgal et al. (46) showed that transforming growth factor (TGF)-β1, whose production is enhanced by hypoxia (38), induced higher secreted levels of MMP-2 through increased

stability of the proenzyme. Expression of $\alpha_v\beta_3$ integrin, an important activator of MMP-2, is reported to be increased in HUVECs exposed to 1% oxygen conditions (52). Asahi et al. (1) reported a significant correlation between the expression of the 150 kDa oxygen-regulated protein, ORP150, and MMP-2 expression, suggesting that ORP150 acts as a molecular chaperone for secreted MMP-2. Vascular endothelial growth factor (VEGF), a pro-angiogenic molecule, is elevated in the serum (2, 5) and plasma (47) of preeclamptic women. It has previously been shown to stimulate release of MMPs from HUVECs (35), retinal microvascular endothelial cells (31), human dermal microvascular endothelial cells (28), and vascular smooth muscle cells (53). The VEGF gene contains a hypoxia inducible factor-1α binding site that is regulated by changes in oxygen (7). In particular, hypoxia upregulates transcription of VEGF (34, 55), which may indirectly stimulate the release of MMPs as previously shown. In our study, varying oxygen mediated a biphasic MMP-2 release from endothelial cells from uncomplicated pregnancies. We initially hypothesized that MMP-2 release would be enhanced at low oxygen (<0.5%, 2%) compared to high oxygen (20%). Interestingly, MMP-2 release was decreased at <0.5% relative to 2% oxygen, possibly due to the lack of a stimulus that regulates oxygen-mediated MMP-2 release. We speculate that 2% oxygen is normoxic for HUVECs and that a shift in the oxygen level in either direction causes decreased MMP-2 release, possibly indirectly through cytokines, growth factors and other biological agents.

As inhibitors of MMPs, the TIMPs function in a variety of contexts to reduce MMP activity. TIMP-1 forms a 1:1 complex with MMP-9 (17) and binds with lower affinity to MMP-2 (33). TIMP-2 forms a complex with MMP-2 (23) and is responsible for both its activation (36) and inhibition (33). The activation of MMP-2 occurs via the formation of a trimolecular complex between membrane-type MMP (MT1-MMP), TIMP-2 and MMP-2 (24) whereby an active MMP-2-TIMP-2 complex may be released (36). The elevated MMP-2 and TIMP-2 release observed in preeclamptic cells may be indicative of the MMP-2 activation process, ultimately leading to increased MMP-2-mediated vascular

reactivity. Alternatively, TIMP-2 elevation may be a compensatory response to the increased MMP-2 release, in order to inhibit enhanced MMP-2-mediated vascular reactivity.

There is a variety of evidence in the literature regarding oxygen regulation of TIMPs. Canning et al. (6) showed decreased TIMP-1 and -2 expression in trophoblasts and decreased TIMP-1 expression in breast cancer cells, exposed to reduced (1%) oxygen conditions. Ben-Yosef et al. (3) showed reduced expression of TIMP-2 mRNA in human macrovascular endothelial cells exposed to hypoxic ($PO_2 = 23 \pm 4$ mm Hg) conditions. In contrast, Eddy (11) demonstrated elevation of TIMP expression in ischemic kidneys and Pardo et al. (41) showed a moderate increase in TIMP-1 in the lungs after hyperoxic (85% oxygen) injury. Our results show that TIMP-1 and -2 secretion from normal cells is not significantly modified by changes in oxygen tension. The diversity of results may be due to differences in cell type, incubation time, method of TIMP detection, and direction of the change in the oxygen environment compared to the physiological level.

In summary, we report that endothelial cells from preeclamptic pregnancies demonstrate significantly enhanced MMP-2, TIMP-1 and -2 release compared to cells from uncomplicated pregnancies. Our data show that there are significant effects of oxygen tension on MMP-2 release from normal cells; however, the magnitude of the enhanced release is small when compared to the differences in MMP-2 release in cells from preeclamptic and uncomplicated pregnancies. Furthermore, TIMP-1 and -2 release is not affected by changes in oxygen. It is unlikely that oxygen is a key mediator of the enhanced MMP-2, TIMP-1 and -2 release observed in preeclamptic cells. We suggest that there are fundamental phenotypic/genotypic differences between endothelial cells from preeclamptic and uncomplicated pregnancies that contribute to the observed differences in MMP-2, TIMP-1 and -2 release.

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Characteristic	Normal Pregnant (n=10)	Preeclampsia (n=4)
Maternal age (years)	26 ± 1.9	25 ± 2.4
Pre-pregnant weight (kg)	72.9 ± 11.9	63.5 ± 4.1
Pre-pregnant BP (mm Hg)	117/71 ± 2/3	110/78 ± 6/5
Term BP (mm Hg)	124/76 ± 4/5	154/111 ± 3/5 *
Proteinuria	N/D	2.5 ± 0.3 *
Gestation (weeks)	38.7 ± 0.5	38.2 ± 0.8
Infant birth weight (g)	3290 ± 148	3217 ± 99

Table 3.1 **Patient demographics of women from Royal Alexandra Hospital, Edmonton, Alberta, from whom umbilical cords were obtained.**

N/D = not detectable; BP = blood pressure; * $P < 0.05$ (vs. normal pregnant women).

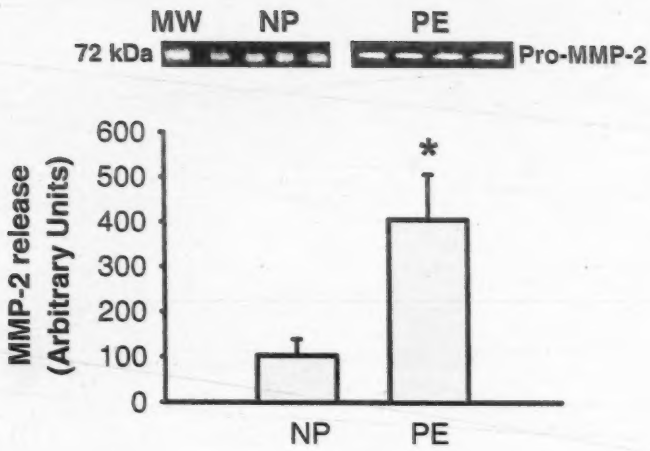

Figure 3.1 Gelatin zymography of MMP-2 release from HUVECs from normal pregnant and preeclamptic women.

Gelatin zymography analysis of MMP-2 release from HUVECs from normal pregnant (NP; n = 4) and preeclamptic (PE; n = 4) women after 12 hours of incubation in Q-media. The upper panel reflects the zymogram (5 μg of protein per lane) showing MMP-2 release. The lower panel shows summary data in arbitrary densitometric units. The data are presented as means ± SEM of the densitometry reading of the zymogram. The MMP-2 zymography standard (Chemicon International, Tenecula, CA) was run concurrently on the gel. * $p < 0.05$

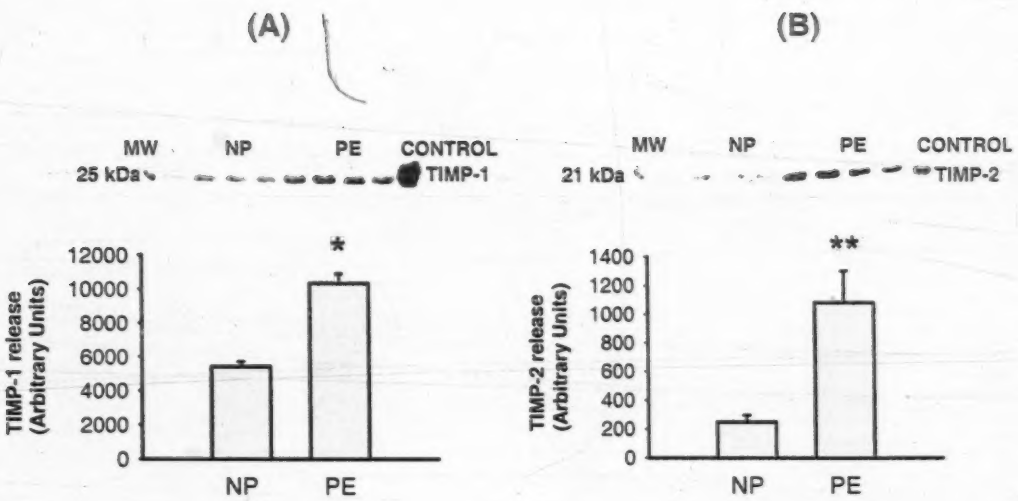

Figure 3.2 Western blot analysis of TIMP-1 and TIMP-2 release from normal pregnant and preeclamptic HUVECs.

Western blot analysis of TIMP-1 **(A)** and -2 **(B)** release from normal pregnant (NP; n = 4) and preeclamptic (PE; n = 4) HUVECs after 12 hours of incubation in Q-media. The upper panel reflects the Western blot (60 μg of protein per lane) showing TIMP release. The lower panel shows the summary data in arbitrary densitometric units. The data are presented as means ± SEM of the densitometry reading of the Western blot. The positive control (30 μg) is concentrated supernatant from HT1080 cells stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA). * $p < 0.001$, ** $p = 0.01$

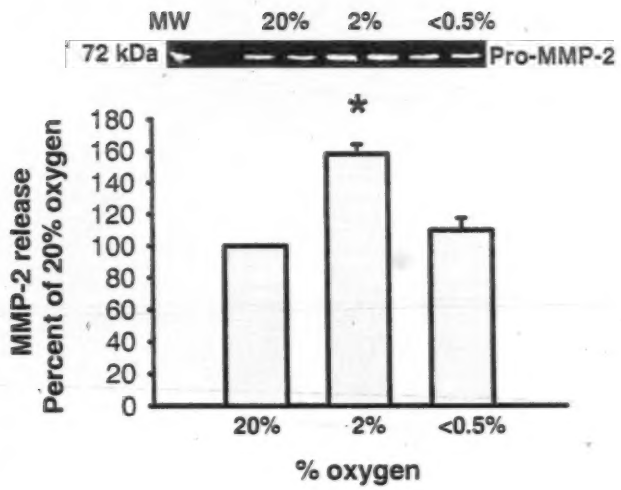

Figure 3.3 Gelatin zymography of MMP-2 release from HUVECs from normal pregnancies under varying oxygen conditions.

Gelatin zymography analysis of MMP-2 release from HUVECs from uncomplicated pregnancies incubated for 12 hours in Q-media at 20% (standard culture conditions), 2% and <0.5% oxygen. The upper panel reflects a representative zymogram (5 μg of protein per lane) of samples from one experiment carried out using cells from one umbilical cord, run on a single gel. The lower panel shows the summary data for all experiments (n = 6). MMP-2 release is presented as a percent of the release at 20% oxygen ± SEM. * $p < 0.05$ for MMP-2 release at 2% oxygen compared to 20% and <0.5%. The MMP-2 zymography standard (Chemicon International, Tenecula, CA) was run concurrently on the gel.

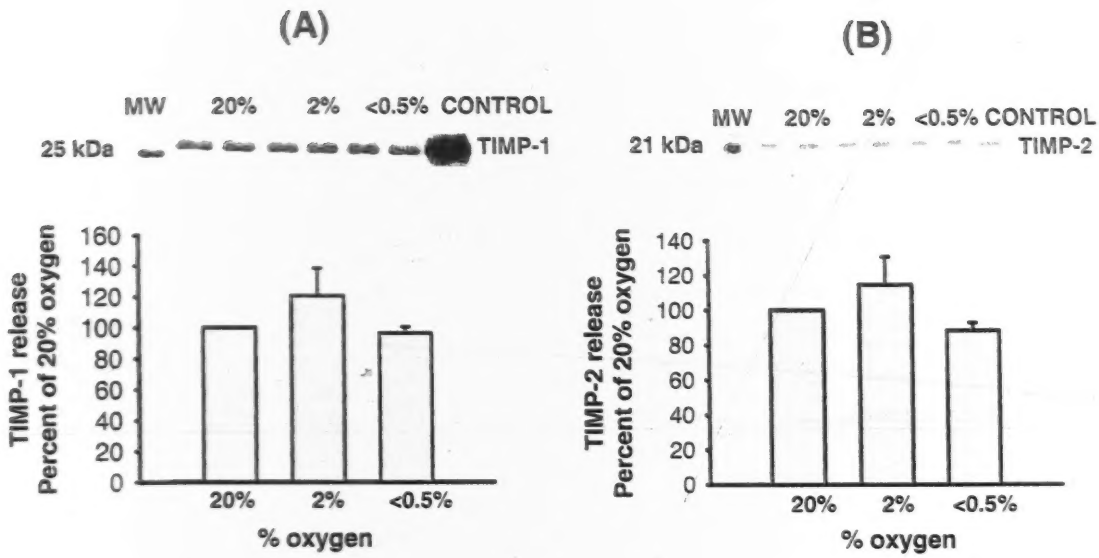

Figure 3.4 TIMP-1 and TIMP-2 release from HUVECs from normal pregnancies under varying oxygen conditions.

TIMP-1 **(A)** and -2 **(B)** release from HUVECs from uncomplicated pregnancies incubated for 12 hours in Q-media at 20% (standard culture conditions), 2% and <0.5% oxygen. The culture supernatants were subjected to Western blot analysis (60 μg of protein per lane) as described in the Methods section. The upper panel shows a representative blot of samples from one experiment carried out using cells from one umbilical cord, run on a single gel. The lower panel shows the summary data for all experiments (n = 4). TIMP-1 (4a) and -2 (4b) release are presented as a percent of the release at 20% oxygen ± SEM. The positive control (30 μg) is concentrated supernatant from HT1080 cells stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA). $p > 0.05$

CHAPTER 4. THE ROLE OF MATRIX METALLOPROTEINASES IN VASCULAR REACTIVITY: IMPLICATIONS FOR NORMAL PREGNANCY AND PREECLAMPSIA.

4.1 INTRODUCTION

Preeclampsia is a multi-system disorder of pregnancy characterized by hypertension and proteinuria (30). There is substantial evidence for a pathogenic model of preeclampsia where insufficient trophoblast invasion of the maternal spiral arteries leads to a poorly perfused fetoplacental unit. This may lead to the secretion of factors into the maternal circulation causing activation of the vascular endothelium (39). In addition, circulating factors may come from sources other than the placenta (5) and maternal response to circulating factors may be heightened in preeclampsia (19); nevertheless, evidence suggests that circulating factors influence vascular reactivity in preeclampsia. Suspected factors include vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF)-α, both of which are expressed in the placenta (5, 25) and alter endothelial cell function (8, 32). Increased levels of VEGF (4, 9, 40) and TNF-α (28) have been reported in the circulation of women with preeclampsia. Another circulating factor that has been implicated is angiotensin II (AII), a potent vasoconstrictor, the levels of which are reduced in preeclampsia compared to normal pregnancy (10, 29); nevertheless, there is heightened pressor sensitivity to AII in preeclampsia (19).

Evidence of circulating factors in women with preeclampsia affecting vascular function has been demonstrated by incubating arteries in the plasma of women with preeclampsia (1, 7, 18, 23). Ashworth et al. (1) and Hayman et al. (23) reported that myometrial

resistance arteries from women with normal pregnancies demonstrated significantly blunted relaxation to the vasodilator, bradykinin, when incubated in the plasma of women with preeclampsia. Brockelsby et al. (7) demonstrated that co-incubation of vessels in the plasma of women with preeclampsia and a VEGF neutralizing antibody, subsequently reversed the impaired relaxation observed in vessels incubated in plasma alone. Similarly, Gandley et al. (18) reported that the impaired relaxation in mesenteric arteries from mice resulting from exposure to plasma from preeclamptic women was reversed by co-incubation with an AT_1 receptor antagonist. These results support roles for the VEGF and AII pathways in the pathophysiology of preeclampsia.

An emerging vascular pathway of interest involves matrix metalloproteinases (MMPs), specifically, MMP-2, the levels of which are elevated in the plasma of women with preeclampsia (35). MMP-2 belongs to a family of extracellular matrix (ECM)-remodeling enzymes and has been shown to mediate vascular reactivity through cleavage of big endothelin (ET) to yield the novel, potent vasoconstrictor, medium ET (15). Furthermore, MMP-2 is capable of cleaving the vasodilatory neuropeptide calcitonin gene-related peptide (CGRP), thus significantly reducing its vasodilatory capacity (16). As a result of these actions, MMP-2 can promote vasoconstriction through production of vasoconstrictors and reduction in levels of vasodilators. VEGF (31, 35), TNF-α (14, 38) and AII (11) are capable of stimulating the production and/or release of MMPs. The role of MMPs as mediators of vascular function in preeclampsia is presently unknown.

One of the mechanisms by which vascular function can be altered is through changes in the myogenic tone of resistance sized arteries. Myogenic tone is an intrinsic property of smooth muscle and is independent of neural, metabolic and hormonal influences. It enables arteries to contract in response to elevations in transmural pressure and dilate in response to reductions in pressure. Two of the most important functions of the vascular myogenic response are the establishment of basal vascular tone and autoregulation of

blood flow and capillary hydrostatic pressure. The mechanisms underlying the myogenic response are very complex; however, there is evidence that there is depolarization of the vascular smooth muscle cell and an increase in intracellular Ca^{2+} (24). Furthermore, myogenic tone is modulated by the endothelium (33), a site of production and release of MMPs (6). The effects of plasma from women with preeclampsia on myogenic tone have only been recently assessed (18) and the role of MMPs as mediators of myogenic tone has not been investigated.

We hypothesized that circulating factors in the plasma of women with preeclampsia would enhance myogenic tone and impair relaxation as compared to plasma from women with normal pregnancies and that this effect would be reversed by inhibition of MMPs.

4.2 MATERIALS AND METHODS

4.2.1 Animals and tissue collection

The evolution of inbred mouse strains and the recent advent of genetically modified mice are being used to gain a better understanding of specific pathways in a variety of syndromes including preeclampsia (13, 34). We conducted our experiments using normal C57BL/6J mice with the hope that further studies examining the role of MMPs in preeclampsia can be conducted in genetically modified mice.

Animal protocols were conducted in accordance with institutional guidelines issued by the Canada Council on Animal Care. Virgin female C57BL/6J mice (4 – 6 months old; Jackson Laboratories; Bar Harbor, ME) were housed in polypropylene cages in groups of

five in a temperature- and humidity-controlled environment. Animal-housing facilities were controlled on a 12 h-12 h light-dark cycle, and mice were given free access to standard laboratory chow and water. After the mice were euthanized (cervical dislocation), the mesentery was removed and placed immediately in freshly prepared cold Dulbecco's medium. Dulbecco's medium contained Dulbecco's Modified Eagle medium base (Sigma) supplemented with 1mM sodium pyruvate, 25mM sodium bicarbonate, 5mM HEPES, and 5mM glucose. We used Dulbecco's medium because it contains nutrient amino acids and vitamins that improved viability of the mouse arteries (42).

Resistance sized (100 μm - 150 μm) mesenteric arteries were dissected free from surrounding fat and connective tissue and transferred to a dual-chamber pressure myograph (Living Systems Instrumentation, Burlington, VT). Vessels were secured between two glass cannulas suspended inside the chamber, after flushing the lumen gently to remove blood. The proximal cannula was connected to a flow-through pressure transducer and pressure servo-control unit. The distal cannula was occluded to prevent flow. Dulbecco's medium was maintained at 37°C and at pH 7.4 in a jacketed water bath.

After mounting, all arteries were examined for ability to maintain pressure. Any decrease in pressure indicated a leak and a new artery was dissected. The second-order mesenteric arteries were equilibrated in warm Dulbecco's medium (which was added to the baths of the system at 10-min. intervals) for 30 min at an intraluminal pressure of 50 mmHg, pre-stretched by increasing intraluminal pressure from 50 to 75 mmHg, and then returned to 50 mmHg (12, 42). The vessels were equilibrated for an additional 30 min at 50 mmHg.

Medium in the dual-chamber arteriograph was maintained at 37°C with the aid of a built-in microprocessor temperature controller that works in conjunction with a power supply.

Minute fluctuations in temperature are detected with a sensitive glass-encased chromel-alumel thermocouple probe inserted into the bath chambers, which is held by clamps alongside the mounted vessels. The vessels were imaged using a video camera, and internal diameter was measured with a video dimension analyzer (21).

4.2.2 Plasma collection protocol

Blood samples were obtained from non-pregnant, normal pregnant, and preeclamptic women at the Royal Alexandra Hospital, Edmonton, Alberta, Canada. The Health Research Ethics Board gave approval for this work and all women who participated gave voluntary, written, informed consent. Preeclampsia was defined using the criteria of hypertension and proteinuria developing after the 20^{th} week of gestation. Hypertension was defined as an absolute blood pressure >140/90 mm Hg on two occasions at least 6 hours apart. Proteinuria was defined as >500 mg/24 hours collection or 2+ on a urine Dipstix test. The women with uncomplicated pregnancies were normotensive throughout gestation and had no proteinuria. No subject was known to have a history of chronic hypertension, liver, renal, or metabolic disease.

Blood samples were collected in a sterile purple-top tube containing ethylenediaminetetraacetic acid (EDTA). The samples were chilled to 4°C and spun in a refrigerated centrifuge at 1600 *g* for 10 mins. The plasma was removed and stored at –80°C.

The individual plasma samples from each group of women (non-pregnant, normal pregnant and preeclamptic) were pooled on the basis of maternal age and maternal pre-pregnant weight. The pooled plasma samples were used in experimental protocols.

4.2.3 Experimental protocols

In the first series of experiments, we evaluated the effects of the MMP inhibitor, GM6001 (Chemicon International, Tenecula, CA), on myogenic tone and on relaxation to the endothelium-dependent vasodilator, methacholine. An initial assessment of myogenic tone was performed on both vessels in the absence of drug in the following manner: 1) the intraluminal pressure was reduced to 10 mmHg after the initial equilibration at 50 mmHg; 2) the pressure was then increased from 10 mmHg to 100 mmHg in 10 mmHg increments, with a diameter measurement being taken 5-6 min after each pressure step. This protocol for assessing myogenic tone was then repeated in the absence and presence of 5 μM GM6001 after a 20 min. pre-incubation (i.e. only one vessel was pre-incubated in GM6001. The other received media only and served as the time control to ascertain repeatability of responses). This concentration of GM6001 was chosen as it is within the range of concentrations previously used in cell culture studies (20, 22). The K_i values for GM6001 are as follows: human MMP-1, 0.4nM; human MMP-3, 27nM; human MMP-2, 0.5nM; human MMP-8, 0.1nM; human MMP-9, 0.2nM (17). After the pressure steps, the vessels were washed with fresh media and were equilibrated at 50 mmHg for 30 mins. Endothelium-dependent vasodilation to methacholine was evaluated in the absence and presence of 5 μM GM6001 (20 min. pre-incubation). The vessels were preconstricted 40 – 60% with increasing doses of phenylephrine. After a steady state constriction was reached, increasing doses of methacholine were added to the bath ($1 \times 10^{-9} - 2 \times 10^{-6}$ M). Diameter readings were taken 3 mins after each addition of methacholine. Vessel relaxation was expressed as a percentage of the preconstricted diameter. Finally, the artery was placed in Ca^{2+}-free buffer with papaverine (10^{-4} M) for evaluation of a passive diameter curve in order to compare vessel responses of the active curve (in the presence of extracellular Ca^{2+}) at the different pressure steps. The comparison between the two curves enables the calculation of myogenic tone at each pressure step.

In another series of experiments, this process for assessing myogenic tone and endothelium dependent relaxation was repeated in vessels that had been preincubated for 1 hour in pooled plasma from individual groups of non-pregnant, normal pregnant, and preeclamptic women in the presence and absence of 5 μM GM6001. Pooled plasma was used at a 2% dilution as this has been previously shown to alter vascular function (23) and activate endothelial cells (2, 3). The 1 hour incubation in plasma was chosen based on a previous study (23). Heparin (1 U/mL) was also added to the baths to prevent the formation of a fibrin clot (23).

4.2.4 Calculations

The percent change in arterial diameter after each 10 mmHg pressure step was calculated using the following equation: Percent change in diameter = $(D_2 - D_1) / D_2 \times 100$, where D_1 is the internal diameter of the vessel at 10 mmHg and D_2 is the internal diameter at the higher pressure (both in the presence of extracellular Ca^{2+}). Percent relaxation was calculated as follows: percent relaxation = $(D_2 - D_{preconstricted}) / (D_1 - D_{preconstricted}) \times 100$ where D_2 is the internal diameter after any given dose of drug, $D_{preconstricted}$ is the internal diameter after preconstriction with phenylephrine and D_1 is the diameter of the vessel prior to preconstriction.

4.2.5 Data analysis

Data are expressed as means ± SE. Analysis of myogenic tone data was performed using two-way repeated-measures analysis of variance (ANOVA) with post hoc Bonferroni's test for multiple comparisons. A Student's t-test was used to test for differences in EC_{50} and maximum relaxation to methacholine. Statistical significance was considered when $p < 0.05$.

4.3 RESULTS

Patient demographics are summarized in **Table 4.1.** Women with preeclampsia had significantly elevated blood pressure at term ($p < 0.001$), evidence of proteinuria, shorter gestation ($p < 0.05$), and gave birth to smaller babies ($p < 0.05$) compared to women with normal pregnancies.

4.3.1 The effects of GM6001 on myogenic tone and endothelium-dependent relaxation

Mesenteric arteries pre-incubated in GM6001 demonstrated significantly enhanced myogenic tone at pressures ≥ 60 mmHg (**Figure 4.1A;** $p < 0.05$) compared to vessels incubated in media only. Vessels pre-incubated in 5 μM GM6001 showed significantly impaired maximum relaxation ($p < 0.001$) and significantly increased EC_{50} (concentration of drug required for 50% relaxation of the vessel; $p < 0.05$) in response to the endothelium-dependent vasodilator, methacholine (**Figure 4.1B**). These data suggest that MMP inhibition blocks vasodilation of the vessel.

4.3.2 The effects of plasma from non-pregnant, normal pregnant, and preeclamptic women on myogenic tone and endothelium-dependent relaxation

Myogenic tone was not significantly altered by pooled plasma from non-pregnant (**Figure 4.2A**) and normal pregnant (**Figure 4.2B**) women. Plasma from women with preeclampsia significantly enhanced myogenic tone at P = 70 mmHg ($p < 0.05$; **Figure 4.2C**) compared to vessels incubated without plasma. Plasma from non-pregnant women significantly impaired vessel relaxation at [methacholine] = 5×10^{-8} M ($p < 0.05$) and there was a trend toward an increased EC_{50} ($p = 0.09$; **Figure 4.3A**). Plasma from normal pregnant women had no significant effect on EC_{50} or maximum relaxation of the vessels as compared to vessels incubated without plasma (**Figure 4.3B**). Plasma from women with preeclampsia significantly decreased the maximum relaxation of vessels as compared to vessels incubated without plasma ($p < 0.05$; **Figure 4.3C**).

4.3.3 The effects of GM6001 on myogenic tone and endothelium-dependent relaxation in vessels incubated in plasma from non-pregnant, normal pregnant and preeclamptic women

The MMP inhibitor, GM6001, did not significantly alter myogenic tone in vessels co-incubated in plasma from non-pregnant (**Figure 4.4A**) and normal pregnant (**Figure 4.4B**) women. However, contrary to our hypothesis, GM6001 significantly enhanced myogenic tone in mesenteric arteries co-incubated in the plasma of women with preeclampsia at P = 100 mmHg ($p < 0.05$; **Figure 4.4C**), suggesting inhibition of a vasodilator. Similarly, GM6001 did not significantly affect relaxation to methacholine in vessels co-incubated in plasma from non-pregnant (**Figure 4.5A**) and normal pregnant (**Figure 4.5B**) women; however, it significantly decreased the maximum relaxation for

vessels co-incubated in plasma from women with preeclampsia ($p < 0.05$; **Figure 4.5C**), again suggesting that MMP inhibition blocked the modulation of a vasodilator.

4.4 DISCUSSION

As MMP-2 has been shown to mediate vascular reactivity, we evaluated the role of MMPs in myogenic tone as well as vasorelaxation. Furthermore, we also studied the effects of pooled plasma from non-pregnant, normal pregnant and preeclamptic women on myogenic tone and endothelium-dependent relaxation and how these properties may be altered by MMP inhibition.

MMP inhibition by GM6001 had significant effects on myogenic tone and vasorelaxation in mesenteric arteries. MMP inhibition enhanced myogenic tone at $P \geq 60$ mmHg, increased the EC_{50}, and blunted the maximum relaxation to methacholine. These results suggest that MMPs are involved in mediating vasodilation. Our laboratory has previously reported MMP-mediated relaxation in which thrombin caused vasodilation of rat mesenteric arteries, associated with enhanced MMP-2 release, and subsequently reversed by the addition of tissue inhibitor of metalloproteinase-2 (TIMP-2). Investigations carried out in other laboratories also support the role of MMPs as mediators of vasodilation. Recently, Novak et al. (36) showed that specific and non-specific MMP inhibitors reversed the reduced myogenic reactivity associated with pregnancy. Furthermore, relaxin, a circulating hormone that mediates vasodilation in the renal vasculature (37) upregulates MMP-2 in small renal arteries (27) and MMP inhibition abolishes the relaxin-induced vasodilation (26). Endothelin, although a potent vasoconstrictor by acting on ET_A receptors on vascular smooth muscle, is also a vasodilator by acting on ET_B receptors on the endothelium (41). Our results suggest that MMPs are involved in mediating vasodilation and reducing myogenic tone, and this may occur in part through

the ET_B receptor pathway. Future studies will examine this possibility. **Figure 4.6** summarizes MMP interactions in the vasculature.

As we hypothesized, incubation of vessels in plasma from women with preeclampsia significantly enhanced myogenic tone, but only at P = 70 mmHg. Plasma from non-pregnant and normal pregnant women did not alter myogenic tone, although vessels incubated in plasma from non-pregnant women displayed behaviour similar to that of vessels incubated in the plasma from women with preeclampsia (i.e. the curves were similar in shape, although statistical significance was not reached). Our results also indicate that pooled plasma from non-pregnant and preeclamptic women significantly impairs endothelium-dependent relaxation, implying that factors in the circulation impair vascular function or alternatively, plasma from normal pregnant women confers vascular protection. These results support those of Gandley et al. (18) who reported that plasma from women with preeclampsia significantly enhanced myogenic tone and blunted the maximal relaxation to methacholine in mesenteric arteries from mice. However, our results are novel in that we have demonstrated that plasma from non-pregnant women also impairs relaxation. This observation is important because it de-emphasizes the role of a poorly perfused placenta as the source of circulating factors that impair vascular reactivity; rather, it further supports the concept that normal pregnancy confers vascular protection. At the present time, differences in myogenic tone are not significant, possibly due to low numbers (n = 3) in the non-pregnant group. Whereas Gandley et al. (18) reported reduced myogenic tone in vessels exposed to plasma from normal pregnant women, we observed no differences in myogenic tone or endothelium-dependent relaxation, implying, in both studies, the presence of protective factors that may mediate the release of vasodilatory substances from the endothelium.

Contrary to our hypothesis, co-incubation with GM6001 failed to reverse the enhanced myogenic tone and blunted relaxation observed in vessels incubated in the plasma of

women with preeclampsia. In fact, myogenic tone was further elevated and relaxation was further blunted in these vessels. This suggests that MMPs play a greater role in modulating myogenic tone and relaxation in vessels exposed to plasma from preeclamptic pregnancies. We speculate that there is a greater degree of MMP-mediated vasodilation in pregnancies complicated by preeclampsia, possibly due to circulating levels of VEGF, TNF-α and AII, all of which stimulate the release of MMPs. The lack of vasoconstrictive effects in vessels exposed to the plasma of non-pregnant and normal pregnant women in the presence of GM6001 imply that MMP-mediated vasodilation due to circulating factors plays a minimal role in the non-pregnant and normal pregnant states.

In conclusion, we report that MMP inhibition significantly enhances myogenic tone and impairs vasorelaxation in mesenteric arteries, suggesting, contrary to our hypothesis, that MMPs are involved in mediating vasodilation. Incubation of mesenteric arteries in the plasma of women with preeclampsia significantly enhanced myogenic tone and impaired relaxation to methacholine, and contrary to our hypothesis, GM6001 failed to reverse these effects. In fact, it further enhanced myogenic tone and abrogated relaxation, suggesting that MMPs may play a greater role in mediating vasodilation in preeclamptic pregnancies. Finally, we have demonstrated for the first time that plasma from non-pregnant women significantly impairs relaxation, suggesting a balance that favours the action of vasoconstrictors. Factors in the plasma of non-pregnant women may also modulate myogenic tone; however, further experiments need to be performed in order to confirm this preliminary observation.

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Characteristic	Non-pregnant (n = 9)	Normal Pregnant (n = 6)	Preeclamptic (n = 12)
Maternal age (years)	27.1 ± 2.9	30 ± 3.1	26.3 ± 1.8
Pre-pregnant weight (lbs)	134.0 ± 5.1	157.2 ± 11.8	171.5 ± 13.1
Pre-pregnant BP (mm Hg)	110/67 ± 4/5	116/72 ± 2/3	115/72 ± 3/3
Term BP (mm Hg)	N/A	121/78 ± 3/3	162/106 ± 3/2 *
Proteinuria (Dipstix)	N/A	N/D	2.7 ± 0.3
Gestation (weeks)	N/A	39.0 ± 0.3	34.9 ± 1.0 *
Infant birth weight (g)	N/A	3590 ± 146	2519 ± 281 *

Table 4.1 Patient demographics of women from Royal Alexandra Hospital, Edmonton, Alberta, from whom plasma samples were obtained.

Summary of patient data from whom plasma samples were obtained at the Royal Alexandra Hospital, Edmonton, Alberta, Canada. BP = blood pressure; N/A = not applicable; N/D = not determined; * $P < 0.05$.

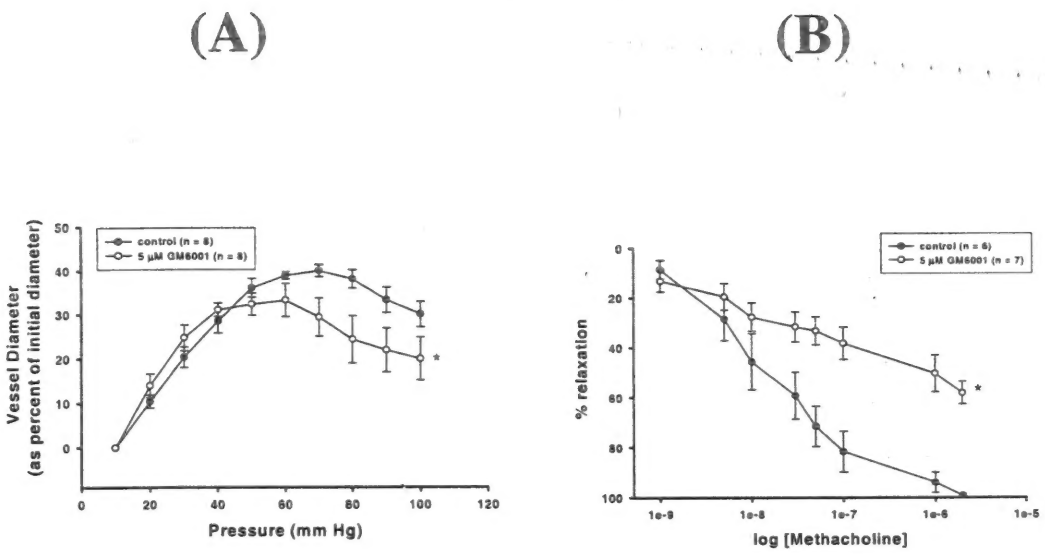

Figure 4.1 The effect of GM6001 on myogenic tone and endothelium-dependent relaxation in mesenteric arteries.

(A) Vessels were pre-incubated in the presence (n = 8) and absence (n = 8) of 5 μM GM6001 for 20 mins. Myogenic tone was enhanced (increased vessel constriction with increasing intraluminal pressure) in vessels incubated in GM6001 at $P \geq 60$ mmHg ($p < 0.05$). Each point represents the mean ± S.E.

(B) Vessels were pre-incubated in the presence (n = 7) and absence (n = 6) of 5 μM GM6001 for 20 mins. Incubation in 5 μM GM6001 significantly impaired the maximum relaxation ($p < 0.001$) and significantly increased the EC_{50} ($p < 0.05$) compared to control vessels. Relaxation to methacholine is expressed as a percentage of the preconstricted diameter. Each point represents the mean ± S.E.

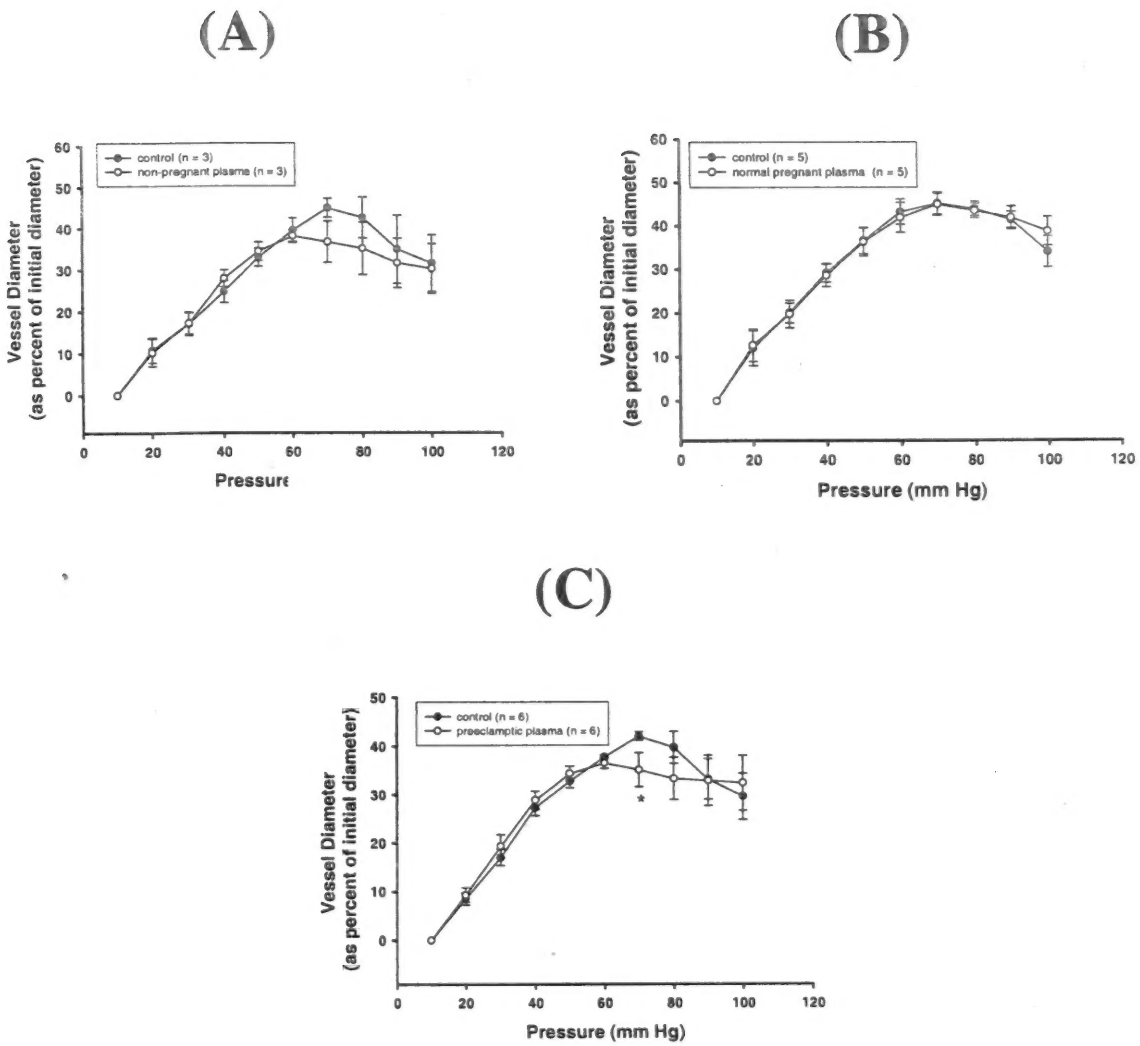

Figure 4.2 The effect of 2% pooled plasma from non-pregnant, normal pregnant and preeclamptic pregnancies on myogenic tone in mesenteric arteries (1 hour pre-incubation).

There were no significant effects of pooled plasma from non-pregnant (**A**; n = 3) and normal pregnant (**B**; n = 5) women on myogenic tone ($p > 0.05$). Plasma from women with preeclampsia (**C**; n = 6) significantly enhanced myogenic tone at P = 70 mmHg ($p < 0.05$). Each point represents the mean ± S.E.

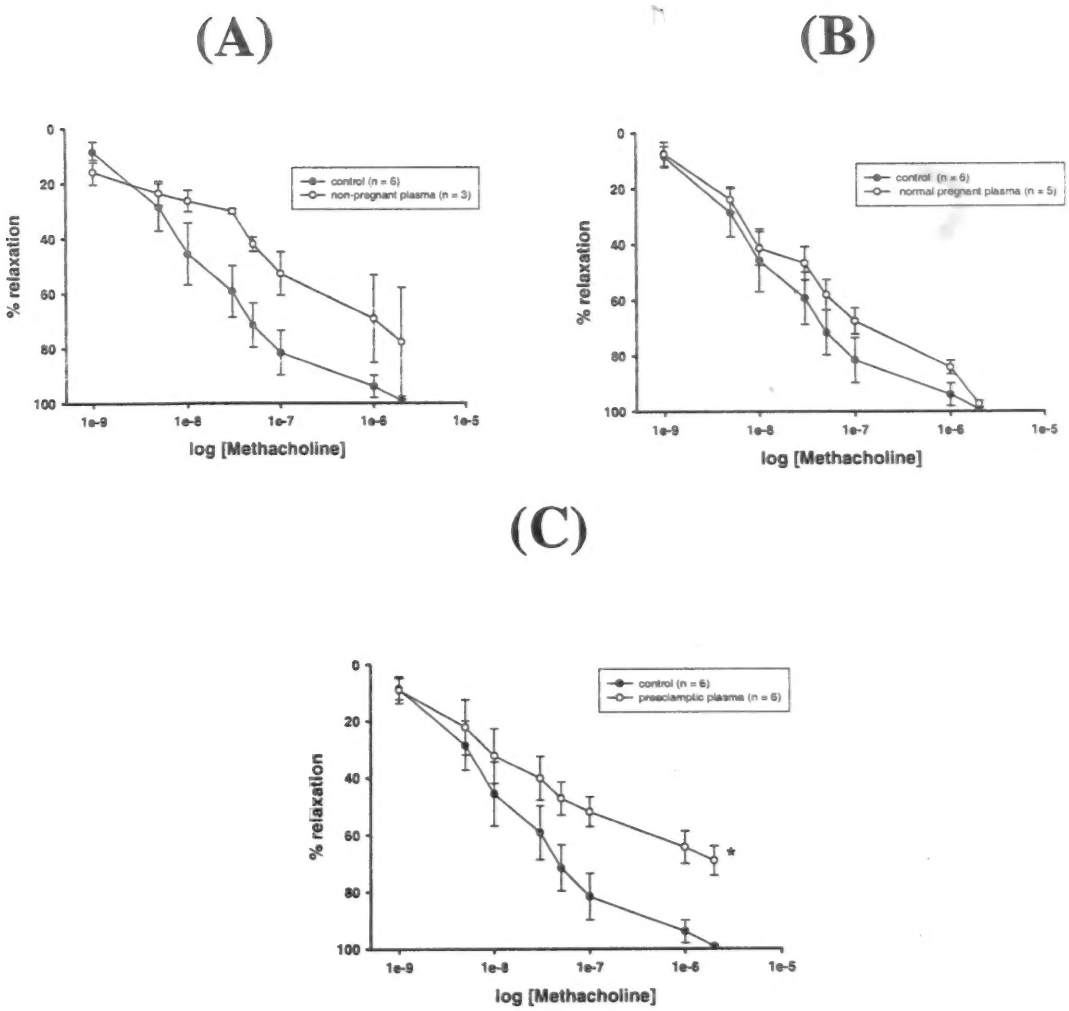

Figure 4.3 The effect of 2% pooled plasma from non-pregnant, normal pregnant and preeclamptic pregnancies on endothelium-dependent relaxation to methacholine in mesenteric arteries (1 hour pre-incubation).

Pooled plasma from non-pregnant women (**A;** n = 3) significantly impaired vessel relaxation at [methacholine] = 5×10^{-8} M ($p < 0.05$) and there is a trend toward an increased EC_{50} ($p = 0.09$). Pooled plasma from normal pregnant (**B;** n = 5) women did not significantly impair relaxation ($p > 0.05$). Pooled plasma from preeclamptic women (**C;** n = 6) significantly blunted the maximum relaxation to methacholine ($p < 0.05$) in mesenteric vessels. Each point represents the mean ± S.E.

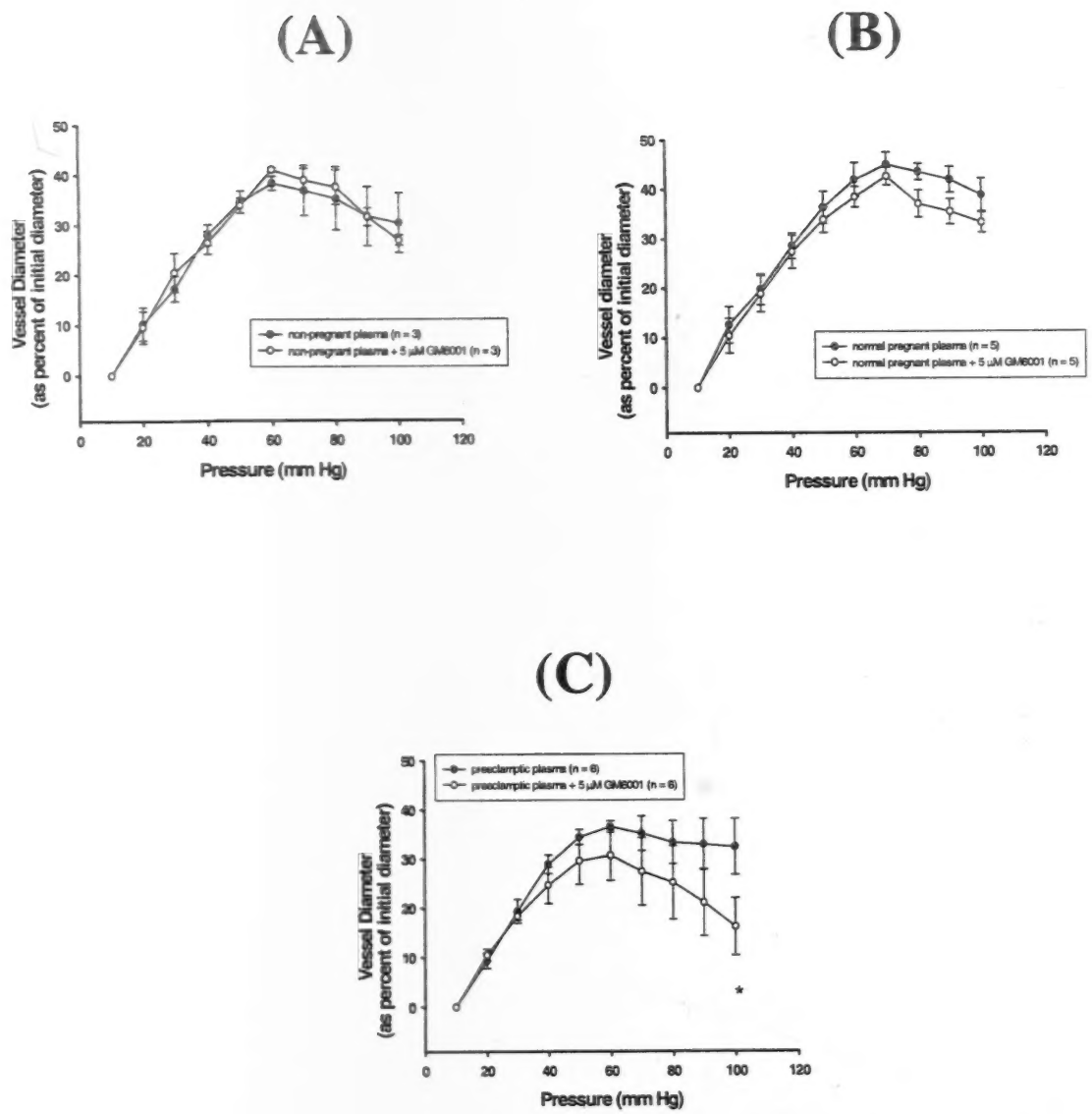
(A)
(B)
(C)
Vessel Diameter (as percent of initial diameter)
Vessel diameter (as percent of initial diameter)
Pressure (mm Hg)
non-pregnant plasma (n = 3)
non-pregnant plasma + 5 μM GM6001 (n = 3)
normal pregnant plasma (n = 5)
normal pregnant plasma + 5 μM GM6001 (n = 5)
preeclamptic plasma (n = 6)
preeclamptic plasma + 5 μM GM6001 (n = 6)

Figure 4.4 The effect of GM6001 on myogenic tone in vessels co-incubated in the plasma of non-pregnant, normal pregnant and preeclamptic women (1 hour pre-incubation).

There was no significant effect of GM6001 on myogenic tone in vessels co-incubated in the plasma of non-pregnant (**A;** n = 3) and normal pregnant (**B;** n = 5) women. GM6001 significantly altered myogenic tone in vessels incubated in the plasma of preeclamptic women ($p < 0.05$; **C;** n = 6) at P = 100 mmHg. Each point represents the mean ± S.E.

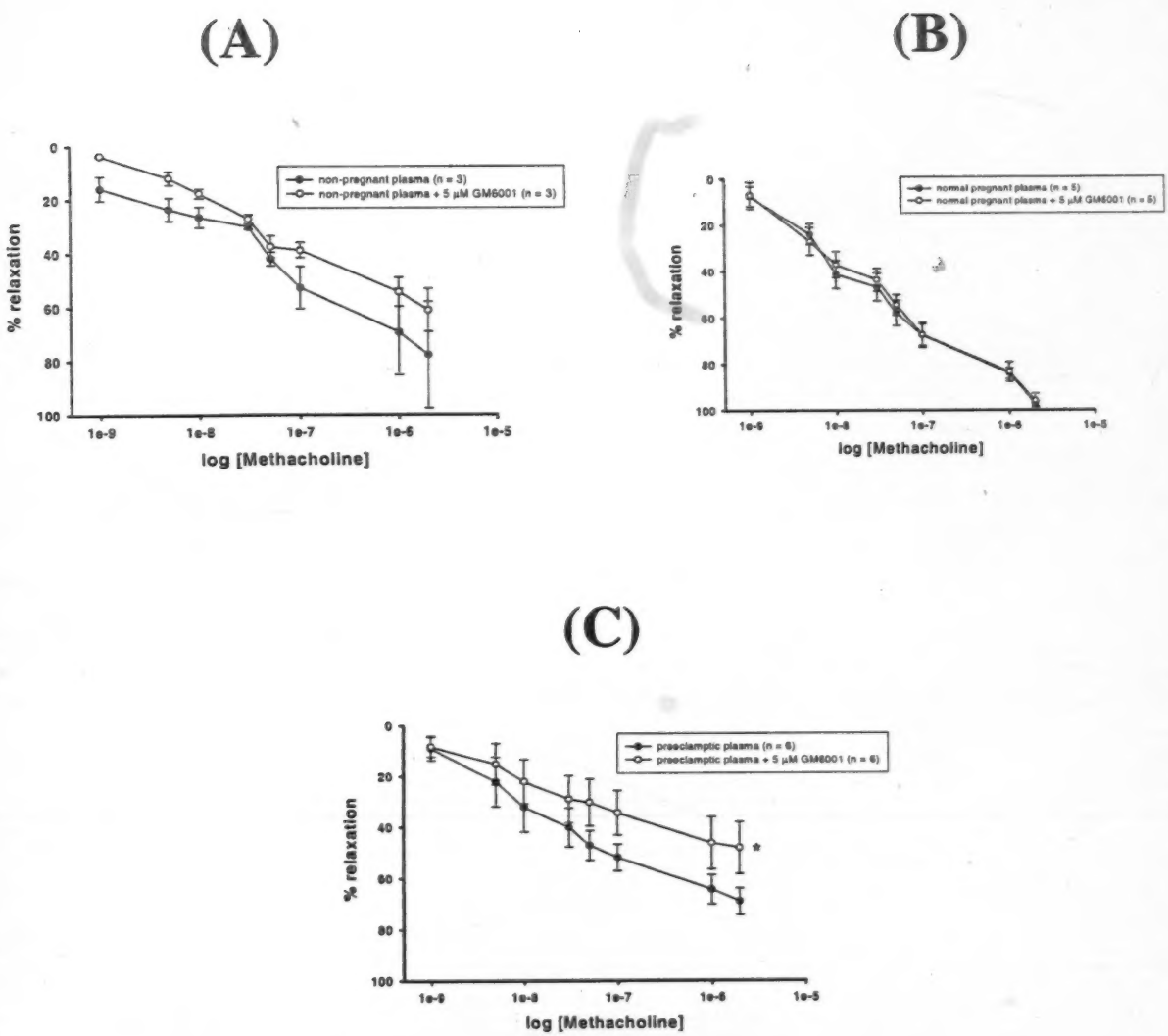

Figure 4.5 The effect of GM6001 on endothelium-dependent relaxation in vessels co-incubated in the plasma of non-pregnant, normal pregnant and preeclamptic women (1 hour pre-incubation).

There was no significant effect of GM6001 on endothelium-dependent relaxation in vessels co-incubated in the plasma of non-pregnant (**A;** n = 3) and normal pregnant (**B;** n = 5) women. GM6001 significantly impaired the maximum relaxation to methacholine in vessels incubated in the plasma of preeclamptic women ($p < 0.05$; **C;** n = 6). Each point represents the mean ± S.E.

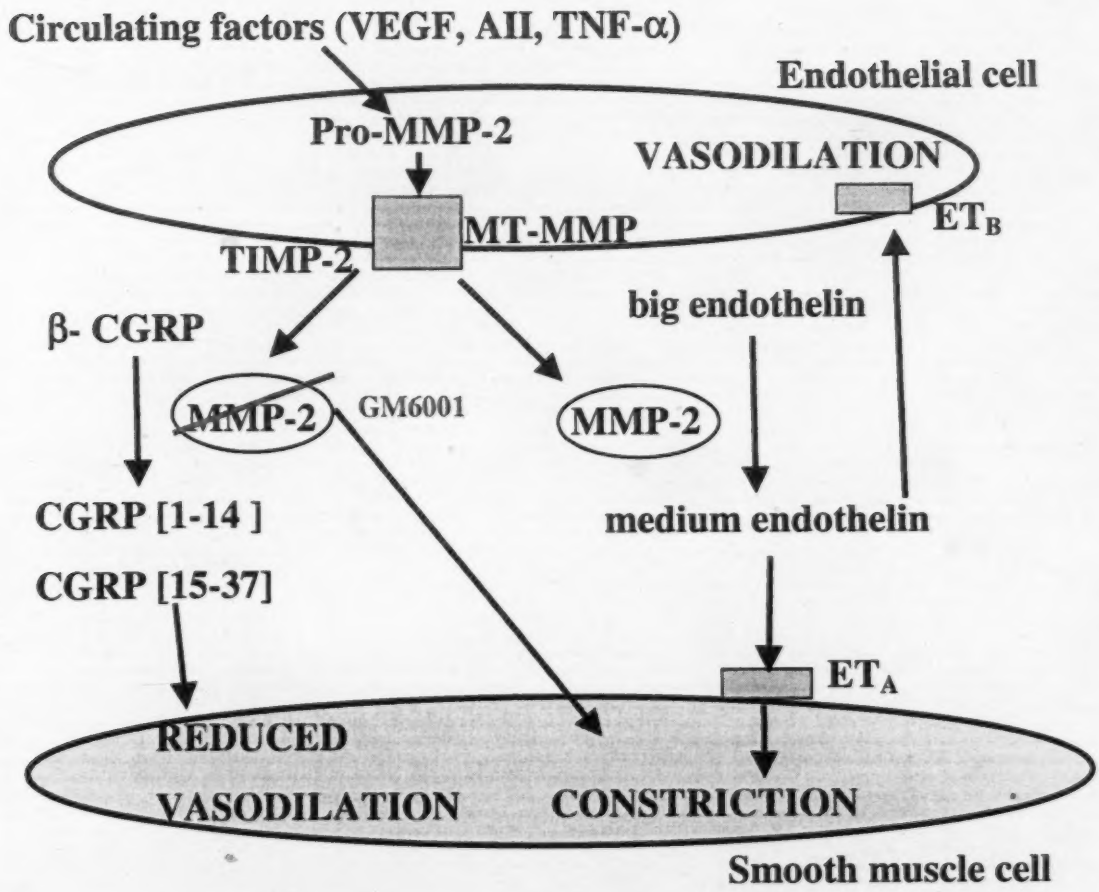

Figure 4.6 MMP interactions in the vasculature.

Pro-MMP-2 (upregulated by VEGF, AII, TNF-α and other circulating factors) is activated at the surface of the endothelial cell by interactions with membrane-type MMPs (MT-MMP) and possibly, tissue inhibitor of metalloproteinase (TIMP)-2. Once activated, MMP-2 can cleave calcitonin gene-related peptide (CGRP), thus significantly decreasing its vasodilatory capacity. Furthermore, MMP-2 can cleave big-endothelin to yield endothelin peptides which can then act on ET_A receptors in the smooth muscle to cause vasoconstriction and/or act on ET_B receptors on the endothelium to mediate vasodilation. In our studies, inhibition of MMPs with GM6001 resulted in enhanced vasoconstriction, implying that MMPs mediate vasodilation in the vasculature, possibly through the ET_B receptor pathway.

CHAPTER 5. GENERAL DISCUSSION.

5.1 SUMMARY

The major focus of this thesis is the study of the role of MMPs in pregnancies complicated by preeclampsia and/or IUGR, specifically, with respect to placental remodeling and altered vascular reactivity. These aspects were studied in 3 separate models: placental explants, cultured endothelial cells (HUVECs) and isolated arteries from mice.

While cellular proliferation and death are quite well characterized in placentas from complicated pregnancies (22, 23), the intricacies of tissue remodeling remain incompletely understood. Using placental explants from normal pregnancies and those complicated by IUGR, we demonstrated reduced release of MMP-2, MMP-9, and TIMP-1 in placental explants from pregnancies complicated by IUGR at high oxygen. These results suggest that the placental maldevelopment in IUGR is more likely to occur under conditions of hyperoxia. We also investigated whether fetal-derived HUVECs from preeclamptic pregnancies demonstrated altered release of MMPs and TIMPs compared to cells from normal pregnancies, and whether this behaviour may be mediated by changes in environmental oxygen tension. Indeed, endothelial cells from pregnancies complicated by preeclampsia demonstrated increased release of MMP-2, TIMP-1 and TIMP-2 compared to cells from normal pregnancies, suggesting that cells from the fetoplacental compartment of preeclamptic pregnancies also demonstrate endothelial activation. Contrary to our hypothesis, exposure of endothelial cells from normal pregnancies to low oxygen levels failed to mimic the behaviour displayed by cells of preeclamptic origin, suggesting that oxygen is not a key mediator of the altered MMP and TIMP secretory

profile in preeclamptic cells. Finally, we studied the role of MMPs as mediators of vascular reactivity in isolated arteries from normal mice exposed to plasma from non-pregnant, normal pregnant, and preeclamptic women. We demonstrated that MMPs are involved in mediating vascular reactivity, specifically, acting as vasodilators in modulating myogenic tone and endothelium-dependent relaxation in the mesenteric vasculature. Plasma from women with preeclampsia significantly enhanced myogenic tone and impaired endothelium-dependent vasodilation in isolated arteries and, contrary to our hypothesis, MMP inhibition failed to reverse these effects. In fact, myogenic tone was further enhanced and endothelium-dependent vasodilation was further impaired, suggesting a greater degree of MMP-mediated vasodilation in preeclamptic pregnancies compared to the non-pregnant and normal pregnant states.

5.2 ALTERED LEVELS OF MMPS AND THEIR INHIBITORS IN PLACENTAL EXPLANTS FROM PREGNANCIES COMPLICATED BY IUGR

MMPs are critical mediators of tissue remodeling in various physiological and pathophysiological processes. We hypothesized that lower levels of MMP-2 and MMP-9 would be released from placental explants from IUGR pregnancies, thus contributing, in part, to the impaired placental development observed in these pregnancies. Interestingly, decreased release of MMP-2 and MMP-9 was only observed under conditions of high oxygen in explants from IUGR pregnancies, suggesting that pathological changes in the placenta may likely occur under conditions of placental intervillous hyperoxia. These observations are supported by recent studies that show increased uterine vein PO_2, but decreased umbilical vein PO_2 and uterine oxygen extraction in IUGR pregnancies (21).

In this study we used the MMP and TIMP secretory profiles as indicators of tissue remodeling within the placenta. Although placental size is reduced in pregnancies

complicated by IUGR (1, 14) and we speculated that impaired tissue remodeling may contribute to this, our work would be stronger if supported by measurements of placental mass. The placental explant experiments were performed in the United Kingdom where it is no longer customary to weight the placenta at delivery, as this measurement is viewed to be grossly inaccurate (23). The placental weight can be an underestimate due to dehydration, or an overestimate due to contamination with amniotic fluid, maternal blood, and fetal blood; consequently, the weights of placentas used in this study were not measured. Despite these problems, some form of assessment of placental size would be useful in the interpretation of the data in relation to tissue remodeling in the placenta.

5.3 THE PLACENTAL EXPLANT MODEL

Placental samples were collected and the placental explant experiments were performed in Manchester, United Kingdom. In this placental explant model, oxygen tension was maintained at 20% and 3% levels, the 3% oxygen atmosphere generated with a control chamber with purge airlock and forced air incubator. In the model, villous tissue was placed on a mesh support in enough culture medium to form a thin layer over the exterior of the tissue. This enables oxygen diffusion through the thin layer of medium and in to the villous tissue. Unfortunately, with this set-up it is difficult to ascertain oxygen levels in the interior compared to the exterior of the explant. We chose to conduct experiments at 20% and 3% oxygen tensions, as we wanted to simulate conditions of hyperoxia and hypoxia in the placenta; however, it is very likely that the interior of the placental explants experienced oxygen levels that were far lower than those experienced by the exterior. At this point in time we do not possess the technology to assess oxygen levels in the interior of explant cultures.

5.4 ENDOTHELIAL ACTIVATION IN CELLS FROM THE FETOPLACENTAL COMPARTMENT OF PREECLAMPTIC PREGNANCIES

In this study we reported that HUVECs from preeclamptic pregnancies demonstrate significantly increased MMP-2, TIMP-1 and TIMP-2 release compared to cells from normal pregnancies, suggesting that even the cells of the fetoplacental compartment experience endothelial activation in preeclamptic pregnancies. Low oxygen levels in the intervillous space due to inadequate adaptation of the uterine vasculature and/or decreased oxygen extraction by the fetus, result in low umbilical vein PO_2 content. If the endothelial cells of the umbilical vein are constantly exposed to these unfavourable conditions, there may be changes at the levels of gene transcription and protein translation, such that even when the cells are isolated and cultured for 3 passages, they still possess a "memory" of the their condition *in vivo*. Indeed, others have also shown that HUVECs from preeclamptic pregnancies demonstrate altered endothelial function (6, 19). Davidge et al (5) reported that the cord blood of infants from preeclamptic pregnancies contained increased levels of cellular fibronectin (an indicator of endothelial cell activation) and that cells exposed to the plasma of infants of preeclamptic mothers produced significantly elevated levels of nitrite compared to cells exposed to the plasma of infants from normal mothers. These results suggest that markers of endothelial cell activation (fibronectin) and endothelial activators are present in the blood of infants of preeclamptic women. The question then becomes, where do markers of endothelial activation and/or endothelial activators in fetal blood come from? It is unlikely that factors in the maternal circulation can cross the placental barrier and enter the fetal circulation. For example, large cellular fibronectin molecules (molecular weight > 200 kDa for an individual subunit) are unlikely to cross the placenta. A more likely possibility is that under-perfusion of the fetoplacental compartment makes the fetus hypoxic, which, in turn, may produce its own circulating factors that cause endothelial activation. Very few studies have examined circulating factors in the cord blood of infants of preeclamptic mothers. While studies have examined fibronectin (5, 10) and TNF-α (2), it would be

interesting to see if other circulating factors may also be involved. In addition, given that endothelial activation is present in the infants of mothers with preeclampsia, it would be intriguing to assess vascular reactivity in fetal vessels in experimental models of preeclampsia. Our laboratory has embarked on this endeavour and is currently investigating the effects of maternal hypoxia in pregnant rats on vascular reactivity of fetal carotid arteries.

We also hypothesized that oxygen may be a critical regulator of MMP and TIMP release from HUVECs and that conditions of low oxygen would cause HUVECs from normal pregnancies to behave like those from preeclamptic pregnancies. Indeed, 2% oxygen increased secretion of MMP-2 from normal HUVECs, but the magnitude of the enhanced release was not as large as that observed in HUVECs from preeclamptic pregnancies. Furthermore, 2% oxygen failed to influence TIMP-1 and TIMP-2 release from normal HUVECs. We thus concluded, that oxygen is not a key regulator of the enhanced MMP-2, TIMP-1 and TIMP-2 release observed in HUVECs of preeclamptic origin and it is likely that these cells are fundamentally different than those from normal pregnancies.

5.5 OXYGEN-MEDIATED MMP/TIMP RELEASE IN PLACENTAL EXPLANTS AND HUVECs: CONFLICTING RESULTS?

It is interesting that the studies examining oxygen-mediated MMP/TIMP release in placental explants and HUVECs produced conflicting results. In placental explant cultures, low oxygen decreased release of MMP-2 and TIMP-1, whereas, in HUVECs, low oxygen enhanced MMP-2 release, but failed to influence TIMP-1 and TIMP-2 secretion. We suggest that our 2 models (placental explants and HUVECs) represent distinct structures within the placenta. For example, MMP secretion in intervillous remodeling (represented by placental explants) may be quite different from that in

intravillous remodeling (represented by endothelial cell cultures). Given their unique placement within the complex structure of the placenta, the 2 models may also be differentially regulated by oxygen, as supported by our results.

These observations highlight the incredible variability in MMP and TIMP response to changes in oxygen tension. From previous studies, and ours, it is evident that MMP/TIMP response to oxygen depends on the direction and duration of the oxygen insult, as well as the tissue and cell type.

5.6 MMPS REDUCE MYOGENIC TONE AND ENHANCE ENDOTHELIUM-DEPENDENT VASODILATION IN THE MESENTERIC VASCULATURE

In this study we demonstrated that MMP inhibition results in impaired endothelium-dependent relaxation and enhanced myogenic tone in mesenteric arteries, suggesting that MMPs are involved in mediating vasodilation in the vasculature, specifically, they decrease myogenic tone and increase endothelium-dependent vasodilation. We also reported that plasma from women with preeclampsia and plasma from non-pregnant women significantly impaired endothelium-dependent vasodilation in mouse mesenteric arteries, suggesting increased susceptibility to vasoconstriction due to circulating factors in maternal plasma, or, alternatively, that plasma from normal pregnant women confers vascular protection. Indeed, endothelial cells exposed to the serum of women with preeclampsia secrete significantly more endothelin-1 (a vasoconstrictor) than cells exposed to the plasma of women with normal pregnancies and interestingly, the secretion of endothelin-1 is further increased in the presence of serum from non-pregnant women (20). Moreover, endothelial cells stimulated with serum from non-pregnant women produce significantly less 6-keto-$PGF_{1\alpha}$ (a stable metabolite of prostacyclin) than those

exposed to serum from normal pregnant and preeclamptic women. These results suggest that circulating factors in the serum of preeclamptic and non-pregnant women alter the release of vasodilators and vasoconstrictors from the endothelium, favouring overall vasoconstriction. The identity of the circulating factor(s) in the maternal circulation that causes endothelial dysfunction in preeclampsia is presently unknown. Although VEGF (4) and AII (12) have been implicated, numerous other candidate factors have been proposed including TNF-α, activated platelets, syncytiotrophoblast microvillous membranes and maternal free fatty acids as substrates for lipid peroxidation (24). The paucity of information pertaining to the deleterious effects of plasma from women with preeclampsia on impaired vascular function (especially with respect to myogenic tone) make it imperative that further studies are conducted in this fascinating area. Currently, our laboratory is investigating how plasma from women with preeclampsia may alter vascular reactivity through TNF-α.

Contrary to our initial hypothesis, the enhanced myogenic tone and impaired endothelium-dependent relaxation in vessels incubated in the plasma of women with preeclampsia were not reversed by MMP inhibition; in fact, myogenic tone was further enhanced and relaxation to methacholine was further impaired. This suggests that MMPs may be involved in mediating vasodilation to a greater degree in preeclamptic pregnancies compared to the non-pregnant and normal pregnant states. This is supported by the elevated levels of MMP-2 in the plasma of women with preeclampsia (17), which may, in fact, be a compensatory response in the face of systemic vasoconstriction. It is possible, however, that women with preeclampsia are more susceptible to MMP inhibition by various circulating factors, leading to impaired vascular reactivity. Indeed, maternal response to circulating factors is increased in preeclampsia (13). In light of this, it would be fascinating to test the effects of MMP inhibitors on isolated arteries from normal pregnant and preeclamptic pregnancies. Furthermore, measuring levels of TIMP-1 and TIMP-2 in the plasma of women with normal pregnancy and preeclampsia may

provide further insight in to the role of MMP inhibitors in the pathophysiology of preeclampsia.

Given the emerging and important role of MMPs in vascular function, it would be interesting to conduct studies on transgenic mice with depleted levels of MMPs (MMP deficient mice). MMP-2 deficient mice have been used to study corneal angiogenesis (16) and tumor progression (15) and MMP-9 deficient mice have been used to study host defence mechanisms in bacterial meningitis (3), and smooth muscle cell migration and arterial remodeling (11). In a similar fashion, transgenic mice models that selectively knock out members of the MMP family may be used to further elucidate the importance of MMPs in vascular reactivity.

Our laboratory has previously shown MMP-2 to have vasoconstrictor properties (8, 9) in endothelial denuded vessels as well as vasodilator properties in endothelium-intact rat mesenteric arteries stimulated with thrombin (7). Our current studies suggest that MMPs are mediators of vasodilation in the mesenteric vasculature of the mouse; however, the mechanism by which this occurs was not explored. Others have shown that MMP-mediated vasodilation may occur through the ET_B receptor pathway (18) on the vascular endothelium in small renal arteries. Our future experiments will address this possibility through selective inhibition of the ET_B receptor in vessels in the absence and presence of plasma from non-pregnant, normal pregnant and preeclamptic women.

5.7 SIGNIFICANCE OF EXPERIMENTS

Although the root of the problem of preeclampsia lies in the placenta, the deleterious effects of that insult are observed in both the fetoplacental compartment and the maternal circulation.

It is likely that decreased levels of ECM-remodeling enzymes under conditions of placental hyperoxia lead to impaired placental development in IUGR pregnancies. In pregnancies that are also complicated by preeclampsia, abnormal uterine vascular adaptation early in pregnancy as well as impaired ongoing placental remodeling may cause placental underperfusion, leading to the secretion of factors that alter endothelial function in the maternal circulation. Furthermore, placental underperfusion may cause fetal hypoxia, and the fetus, in turn, may produce its own circulating factors that cause endothelial activation. This cascade of events is illustrated in **Figure 5.1**.

This thesis presents novel information on the role of MMPs in placental remodeling and vascular reactivity in normal and complicated pregnancies. The focus on oxygen-mediated MMP/TIMP release re-emphasizes the critical role of oxygen in normal pregnancy, the deviation from which may have negative consequences on the cells of the fetoplacental compartment. Furthermore, the studies examining the role of MMPs in vascular function open doors to an intriguing vascular pathway, the inhibition of which may contribute to the pathophysiology of preeclampsia. Future research in this area will expand our understanding of the role of MMPs in normal and complicated pregnancies.

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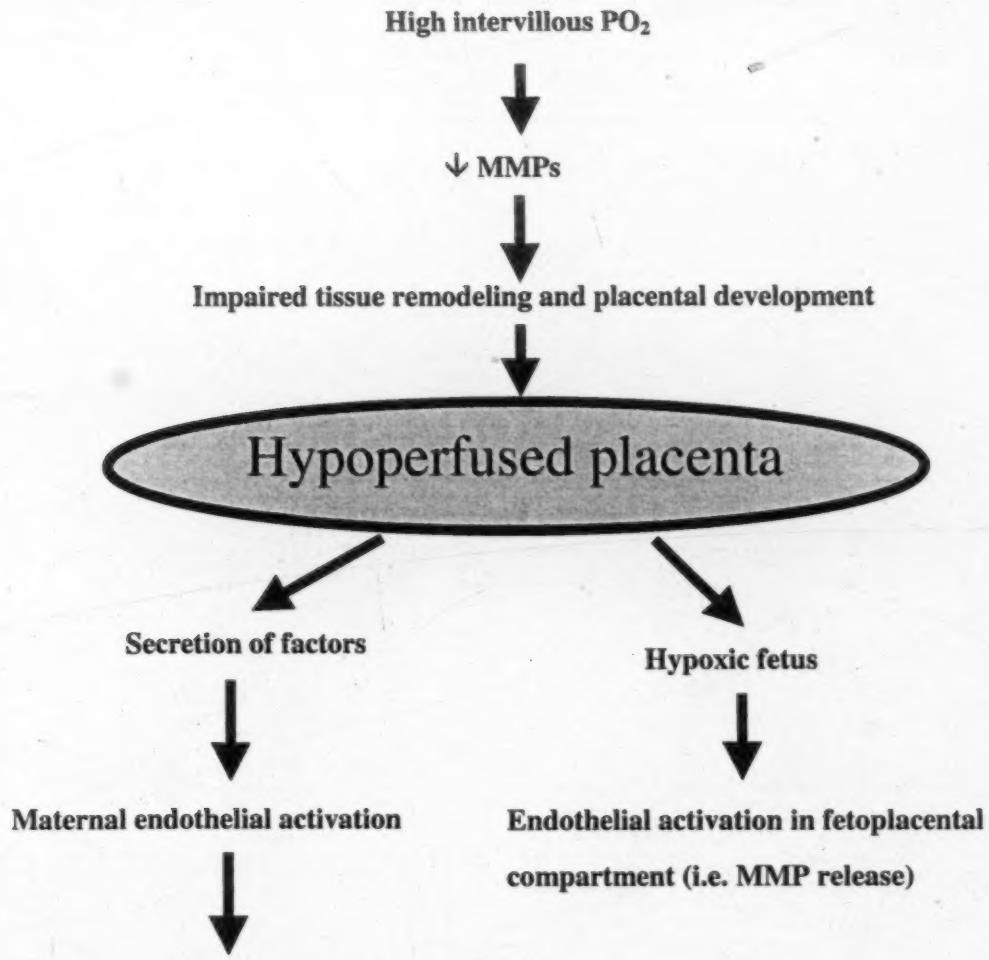

Figure 5.1 The role of MMPs in the pathophysiology of preeclampsia and/or IUGR.

High intervillous PO_2 in IUGR can decrease the release of MMP-2 and MMP-9 from the placenta, and may contribute, in part, to impaired placental development. In pregnancies that are further complicated by preeclampsia, inadequate adaptation of the uterine vasculature early in pregnancy and impaired ongoing placental remodeling, may lead to the formation of a hypoperfused placenta. The hypoperfused placenta can secrete factors that activate the maternal endothelium, ultimately leading to altered vascular reactivity, possibly due, in part, to MMPs. Furthermore, a hypoperfused placenta can cause the fetus to become hypoxic, which may lead to endothelial activation (i.e. increased MMP-2 release) in the cells of the fetoplacental compartment.

I have included appendices in this thesis for two reasons. First of all, I spent a great deal of time and effort optimizing conditions for certain laboratory techniques and feel that this work should be available as a resource for others in my field. Secondly, my preliminary experiments focused on the effects of vascular endothelial growth factor (VEGF) on the stimulation of MMP release from endothelial cells. The data that I obtained from these experiments was variable and confusing. The effect of VEGF on the stimulation of MMP-2 and –9 release was simply not robust enough to accurately quantitate with the available scientific tools. I feel that my work in this area should be recognized, regardless of the fact that the data remains unpublished. Furthermore, my experiences with VEGF enabled me to develop protocols that I feel may be useful to others in my laboratory.

For the sake of simplicity, there are two separate appendices. Appendix A describes Materials/Methods and Results for protocols that I optimized in the laboratory. Appendix B contains the Materials/Methods and Results from selected VEGF experiments.

APPENDIX A: OPTIMIZATION OF EXPERIMENTAL PROTOCOLS

Immunocytochemistry:

As my first project was conducted using human umbilical vein endothelial cells (HUVECs), I ensured that these cultures were immunostained regularly to assess purity, using an antibody against the endothelial-specific von Willebrand Factor (vWF; Sigma). HUVECs were plated at a concentration of 500,000 cells/well in gelatin-coated 6-well plates containing 1 ml of M199 medium supplemented with 20% fetal bovine serum (FBS), 2% penicillin-streptomycin, 1% L-glutamine, and 1% endothelial cell growth

supplement (ECGS). The cells were allowed to adhere to the bottom of the plate overnight. The medium was removed, and cells were washed with cold PBS (3 × 1 ml). Ice-cold methanol (500 µl) was added to each well and the plates were incubated at – 20 °C for 10 minutes. The methanol was removed and the cells were washed 3 times in cold PBS to remove the methanol. PBS was added to each well and the plates were stored at 4 °C until immunostaining.

Upon initiation of the immunostaining protocol, PBS was removed. The cells were blocked in 10% normal goat serum (NGS) for 30 mins at room temperature. The blocking buffer was removed and primary antibody diluted in 4% NGS (50 µg/ml, rabbit anti-human vWF) was added to the cells overnight at 4°C. To serve as a control, rabbit IgG (50 µg/ml, ChromPure Rabbit IgG, whole molecule, Jackson Immunoresearch Laboratories) was also added to the cells. The primary antibody was removed and the cells were washed 3 times with 4% NGS. Secondary antibody (anti-rabbit fluorescein isothiocyanate; Sigma) diluted in 4% NGS was added to the cells for 45 mins at room temperature and the plates were covered with aluminum foil to prevent fluorescence quenching. The cells were washed 3 times with PBS, and DAPI (4,6-diamidino-2-phenylindole, Molecular Probes) diluted in PBS was added to the cells for 10 minutes at room temperature in order to stain all nuclei. Images were taken using a fluorescence microscope (LEICA DM IRB) and the purity of the cultures was assessed visually. **Figure A.1** illustrates HUVECs immunostained with DAPI (**Figure A.1A**) and vWF (**Figure A.1B**) antibody. The rabbit IgG controls stained with DAPI (**Figure A.1C**) and vWF (**Figure A.1D**) are also shown.

Zymography:

Zymography was the key technique I used in assessing levels of MMP-2 and –9 in supernatants from HUVECs and placental explants. The following section will describe this valuable technique in detail.

Zymography was performed according to previously established methods (3, 5). Two advantages of this technique are that both the proenzyme and active forms of MMPs, distinguished on the basis of molecular weight, can be detected (4) and it can be used as a quantitative technique to assess existing and potential gelatinase activity (6). The enzymes are separated under denaturing (sodium docecyl sulphate, SDS) but nonreducing conditions, refolded in a non-ionic detergent that removes the SDS, and then incubated in a suitable buffer system for the enzymes under study (6). While gelatin zymography can detect gelatinases that are active *in vivo*, zymogen forms of MMPs are activated by the process of denaturation (by SDS) and renaturation (by a non-ionic detergent) (8, 9) and are visualized in zymograms along with the active forms. Furthermore, the electrophoretic process effectively separates MMPs from their endogenous inhibitors (9).

Samples containing equal protein amounts (5µg – 10µg) were prepared by dilution in a 6X sample buffer (7 ml 4X Tris-HCl/SDS pH 6.8, 3 ml glycerol, 1g SDS, 1.2 mg bromophenol blue, and water added to 10 ml) and loaded without prior boiling into lanes of a 7.5% polyacrylamide gel containing SDS co-polymerized with 3 mg/ml gelatin. Gelatin functions as an *in situ* protease substrate (4). Samples were electrophoresed at 100 V for 20 minutes and then at 150 V for an additional 50 minutes in running buffer (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS). After electrophoresis the gels were washed in 50 ml of 2.5% Triton X-100 (3 × 20 minutes) and incubated in 100 ml of development buffer (0.15M NaCl, 5mM $CaCl_2$, 0.05% NaN_3, and 50mM Tris-HCl) for 18 - 20 hours at 37 °C. The gels were stained for 1.5 hours in 50 ml Coomassie blue stain (25% methanol, 10% acetic acid, 65% water) and then destained overnight (4% ethanol, 8% acetic acid, 88% water). The clear bands indicating gelatinase activity were scanned

with a densitometer (Fluor-S MAX MultiImager, Bio-Rad Laboratories, CA) and quantitated using the Quantity One Image Analysis System (Bio-Rad Laboratories, CA). We detected primarily latent (pro)-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) in our zymographies. MMP-2 and MMP-9 were identified by comparison with an MMP-2/9 standard (4 µL; human MMP-2/9 zymography standard, Chemicon International, Tenecula, CA) that was run concurrently on the gel.

Sensitivity of the zymography assay:

In order to determine the amount of protein I needed to load to detect differences between my supernatant samples, it was important to assess the sensitivity range of the zymography assay. Increasing volumes (5, 10, 20 µl) of the MMP-2/9 zymography standard (Chemicon International, Tenecula, CA) were loaded in to individual lanes of a gel (**Figure A.2**). The MMP-2 (**Figure A.2A**) and MMP-9 (**Figure A.2B**) bands from the standard were easily observed on the zymogram and MMP-9 was consistently the broadest band, indicating the greatest amount of MMP activity. Furthermore, because all supernatant samples that were analyzed had consistently lower levels of MMP activity compared to the MMP-2/9 standard, we were confident that there was sufficient gelatin in the gel so as to avoid substrate exhaustion. A dose response curve for cell culture supernatant was performed in order to assess the linear range of the zymography assay (**Figure A.2C**) and helped me to determine the amount of protein to use in the analysis of supernatants from experiments.

Levels of endogenous metalloproteinases in serum-containing M199 media:

Fetal bovine serum (FBS) added to media contains MMP-2. It was important for me to determine how much MMP-2 was contained in culture media, so that I could accurately determine the amount of MMP-2 released from HUVECs and placental explants. Zymography was performed on M199 media (GibcoBRL) samples containing increasing percentages of serum (0, 1, 2.5, 5, 10, 20%) in order to evaluate the relative levels of endogenous MMPs. MMP-2 (but not MMP-9) was detected in all FBS-containing samples; however, the levels were far less than those contained in conditioned medium from HUVECs and placental explants. This gave me confidence in my ability to accurately measure release from HUVECs and placental explants without having to worry about high background levels of MMP-2 in media. Media containing 1% FBS was used in HUVEC experiments and 5% FBS in placental explant experiments. **Figure A.3** illustrates the zymogram containing all samples.

MMP-2 accumulation in media over time:

I spent a great deal of time determining the incubation time for my cell culture experiments. I needed to know how long to incubate my cells in order to detect an MMP-2/9 signal in the supernatants that I could accurately quantitate with zymography and densitometry. HUVECs were incubated for 2, 6, and 12 hours under standard culture conditions and demonstrated a time-dependent accumulation of MMP-2 in media (**Figure A.4**). I chose to use the 12 hour incubation time in my subsequent experiments as it enabled me to accurately quantitate MMP activity.

HT1080 cells as positive controls for MMP-2, MMP-9, TIMP-1 and TIMP-2:

When conducting Western blot analysis, it is important to have positive controls to be sure that a particular antibody is working. Since I used Western blot analysis extensively to detect TIMP-1 and –2 in culture supernatants, it was necessary to have appropriate positive controls. I used conditioned medium from human fibrosarcoma HT1080 cells as positive controls for MMP-2 and –9 and TIMP-1 and –2.

HT1080 cells were obtained (Dr. M. Radomski's laboratory, University of Alberta) and grown to confluence in Dulbecco's Modified eagle's Medium (DMEM; Sigma) media supplemented with 10% FBS in a single T25 flask (Costar, Corning, NY). The cells were passaged in to 2 separate T75 flasks (Costar, Corning, NY) and grown to confluence. DMEM was added to both flasks and one flask was stimulated overnight with 100 nM phorbol 12-myristate 13-acetate (PMA). Media was collected from both flasks, and centrifuged at 12,000 rpm for 10 minutes to remove cellular debris. The remaining cells were trypsinized, centrifuged to remove media, and lysed (25 mM Tris-Cl pH 7.5, 0.5% Triton X-100, 1% protease inhibitor, 1% phosphatase inhibitor). Samples were sonicated for 4 – 6 seconds (VibraCell, Sonics and Materials Inc., Danbury, CT) to ensure complete lysis of the cells. Lysates were aliquoted and stored at – 80 ºC until ready to use. 10 ml of media from each group was aliquoted and stored at –80 ºC. The remaining 10 ml of media was concentrated 10X (Millipore, Ultra-free protein concentrator, molecular weight cut off 5000 kDa) and protein concentrations were measured (BCA Protein Assay Kit, Pierce, Rockford, IL). Western blot analysis was performed on all samples (concentrated, unconcentrated, stimulated, unstimulated). The Western blots were probed with antibodies to MMP-2 (1:100 dilution, Santa Cruz Biotechnology, CA), MMP-9 (1:100 dilution, Santa Cruz Biotechnology, CA), TIMP-1 (1:100 dilution, Calbiochem, San Diego, CA) and TIMP-2 (1:100 dilution, Calbiochem, San Diego, CA).

The active (62 kDa) form of MMP-2 was detected in concentrated supernatant from unstimulated cells (**Figure A.5A**). MMP-9 (92 kDa) was detected in conditioned

concentrated medium from cells that had been stimulated with PMA (**Figure A.5B**). TIMP-1 (29 kDa) was detected in all supernatant samples but was the most abundant in conditioned concentrated medium from cells that had been stimulated with PMA (**Figure A.5C**). TIMP-2 (21 kDa) was detected in the conditioned concentrated medium from unstimulated cells (**Figure A.5D**).

APPENDIX B: VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) EXPERIMENTS

3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for HUVEC metabolic capacity:

My initial experiments focused on assessing the bioactivity of the VEGF that was purchased for use in my experiments. Since VEGF is a survival factor for endothelial cells (2), I used the MTT assay to assess HUVEC metabolic capacity in the presence of various concentrations of VEGF. The following protocol was obtained from Dr. L. Guilbert's laboratory (University of Alberta).

Metabolic capacity is proportional to cell number and assessed by the ability of the cells to cleave the tetrazolium salt, MTT, an event that occurs within the mitochondria by the dehydrogenase enzyme and requires energy. In its native state, MTT is a yellow, water soluble substrate but when cleaved it turns into an insoluble blue chromophore which can be dissolved in an organic solvent and quantitated on a plate reader.

HUVECs were plated at 12,000 cells/well (~ 50% confluent) in a 96 well plate in 250 µl of M199 medium (20% FBS, 2% penicillin-streptomycin, 1% L-glutamine, and 1% ECGS). The cells were allowed to adhere to the bottom of the plate overnight. Medium was removed and the cells were carefully washed with 1X Hanks' Balanced Salt Solution (GibcoBRL). Doubling dilutions of VEGF in Q- media (1% FBS) were added to the wells in triplicate. Three wells (with no cells) received Q-media alone, thus serving as the control. The cells were then incubated under standard culture conditions for 24 or 48 hours. The plates were removed from the incubator and 10 µl of the MTT stock solution (5 mg/ml in phosphate buffered saline, filter sterilized and wrapped in aluminum foil to protect from light) was added to all the wells. The plates were placed back in the incubator for 4 hours to allow for the cells to cleave enough MTT to give a good signal to noise ratio. The reaction product (formazan) was then dissolved in 150 µl organic solvent (0.04M HCl in isopropanol) and the solution was mixed vigorously with the multi-channel pipettor. The plates were wrapped in parafilm and incubated at room temperature overnight to allow complete dissolution of the formazan crystals. The optical absorbance was evaluated on a microtiter plate reader (Molecular Devices, UV Max, Kinetic Microplate Reader, Menlo Park, CA) using a test wavelength of 550 nm (A_{550}) and a reference wavelength of 650 nm (A_{650}). At 24 hours and 48 hours, a dose dependent response to VEGF was observed, with maximum cell metabolic capacity in the VEGF-stimulated cultures at a concentration of 10 ng/ml (**Figure B.1**).

Vascular endothelial growth factor (VEGF) stimulation:

Once I had determined that the VEGF was biologically active (using MTT assays), I began experiments to test the effects of VEGF stimulation on MMP-2 release from HUVECs. HUVECs (passages 2 to 3) were propagated on gelatin-coated 6-well dishes at a concentration of 500,000 cells/well. All experiments were performed in duplicate wells. The medium was removed, the cultures were rinsed with Hanks' Balanced Salt Solution

(Gibco) and then incubated for various times under standard culture conditions in Q-media (Gibco, phenol-red free M199 media, 1% FBS, 2% penicillin-streptomycin, 1% L-glutamine), containing $VEGF_{165}$ (Sigma) in a concentration range of 0 – 200 ng/ml. The concentration range of 0 – 10 ng/ml was previously used by our laboratory to stimulate MMP-2 release from HUVECs (7) and the range 50 – 200 ng/ml was further investigated. After 12 hours, the media collected from the cultures was centrifuged at 12,000 rpm for 10 minutes to remove cellular debris and the supernatant was aliquoted into Eppendorf tubes and stored at –20 °C for analysis. Protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL) was used to determine the total protein concentration of the supernatant and lysate samples. The samples were then analyzed using zymography for total gelatinolytic activity. **Figure B.2** illustrates MMP-2 release from HUVECs stimulated with varying concentrations of VEGF. There were no significant effects of VEGF on stimulation of MMP-2 release at all concentrations used.

Anti-human VEGF neutralizing antibody treatment:

Experiments using anti-human VEGF neutralizing antibody (R & D Systems) were conducted in order to evaluate the effects of endogenous and exogenous VEGF on the release of MMPs from HUVECs. In experiments where both exogenous VEGF (Sigma) and neutralizing antibody were added to cultures, the two were pre-incubated together at room temperature for 2 hours prior to addition to cell cultures. The cells were treated as follows: A) Q-media only (control); B) 5 ng/ml VEGF; C) 0.3 μg/ml anti-human VEGF neutralizing antibody; D) 1.0 μg/ml anti-human VEGF neutralizing antibody; E) 5 ng/ml VEGF + 0.3 μg/ml anti-human VEGF neutralizing antibody, pre-incubated together at room temperature for 2 hours; F) 5 ng/ml VEGF + 1.0 μg/mL anti-human VEGF neutralizing antibody, pre-incubated together at room temperature for 2 hours. After a 12 hour incubation under standard culture conditions, supernatants were collected as previously described and zymography was performed. The neutralizing antibody (0.3

μg/ml, 1.0 μg/ml) was found to significantly decrease MMP-2 release in the absence ($p < 0.05$) and presence ($p < 0.05$) of exogenous (5 ng/ml) VEGF (**Figure B.3**).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for VEGF receptors:

I spent a great deal of time trying to detect the VEGF receptors, Flt-1 and KDR/Flk-1, in cell lysates from HUVECs using Western blot analysis. My goal was to study how exposure to exogenous VEGF and/or low oxygen may alter levels of these receptors. Antibodies for these receptors as well as respective blocking peptides were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The Flt-1 and KDR/Flk-1 antibodies were used at a 1:100 dilution and their respective blocking peptides were used at a 1:10 dilution. The antibody and blocking peptide were pre-incubated together at room temperature for 2 hours prior to adding the mixture to the membrane. Despite many attempts to detect these receptors, I was unsuccessful; consequently, I turned to RT-PCR analysis to assess receptor mRNA levels. Dr. S. Fang (Perinatal Research Centre, University of Alberta) provided valuable technical expertise during this endeavour.

Cells were incubated for 12 hours under standard culture conditions. Total RNA was extracted using the TRIzol method (Invitrogen Life Technologies) and RNA was pooled from three identical wells. Chloroform (0.2 ml) was added, the tube was shaken vigorously by hand for 20 seconds, incubated at room temperature for 3-5 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The aqueous layer was transferred to a clean Eppendorf tube, an equal volume of isopropanol was added, the tube was inverted gently 6 times to mix, and then placed at –80 °C for 1.5 hours. The tube was centrifuged at 14,000 rpm for 15 minutes at 4 °C at which point a white-yellow pellet was visible. The supernatant was carefully removed, 1 ml of 75% ethanol was added to the pellet, the

tube was centrifuged at 10,000 rpm at 4 °C for 5 minutes, the supernatant was removed and the pellet was allowed to air dry at room temperature for 5 – 10 minutes. The RNA pellet was dissolved in 10 – 100 µl Tris-EDTA and its concentration was determined by spectrophotometer (A_{260}).

50 ng of total RNA was added to a 50 µl RT-PCR reaction (QIAGEN OneStep RT-PCR Kit). The reaction master mix was prepared according to the manufacturer's protocol to give final concentrations of 1X QIAGEN OneStep RT-PCR Buffer, 400 µM of each dNTP, 0.6 µM of each primer, 2.5 mM $MgCl_2$, and 2 µl/reaction QIAGEN OneStep RT-PCR enzyme mix. Sequences of oligonucleotide primers were obtained from Gerber et al. (1) and primers were synthesized by Sigma Genosys (Oakville, Ontario). The sequences of nucleotides used are in **Table B.1**. The primers for the human KDR/Flk-1 gene were HUMKDR 2530.F and HUMKDR 3043.R. For Flt-1 analysis the primers were HSFLT 2689.R and HSFLT 2228.F.

RT-PCR reactions were monitored by an iCycler Version 1.280 (Bio-Rad Laboratories, CA). Conditions were as follows: 1 cycle at 50 °C for 30 minutes, 1 cycle at 95 °C for 15 minutes, 40 cycles at 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute, and 1 cycle at 72 °C for 10 minutes.

We confirmed the presence of Flt-1 and KDR/Flk-1 in HUVECs (**Figure B.4**). The KDR amplicon (513 kb) was present in lower quantities compared to the Flt-1 amplicon (461 kb).

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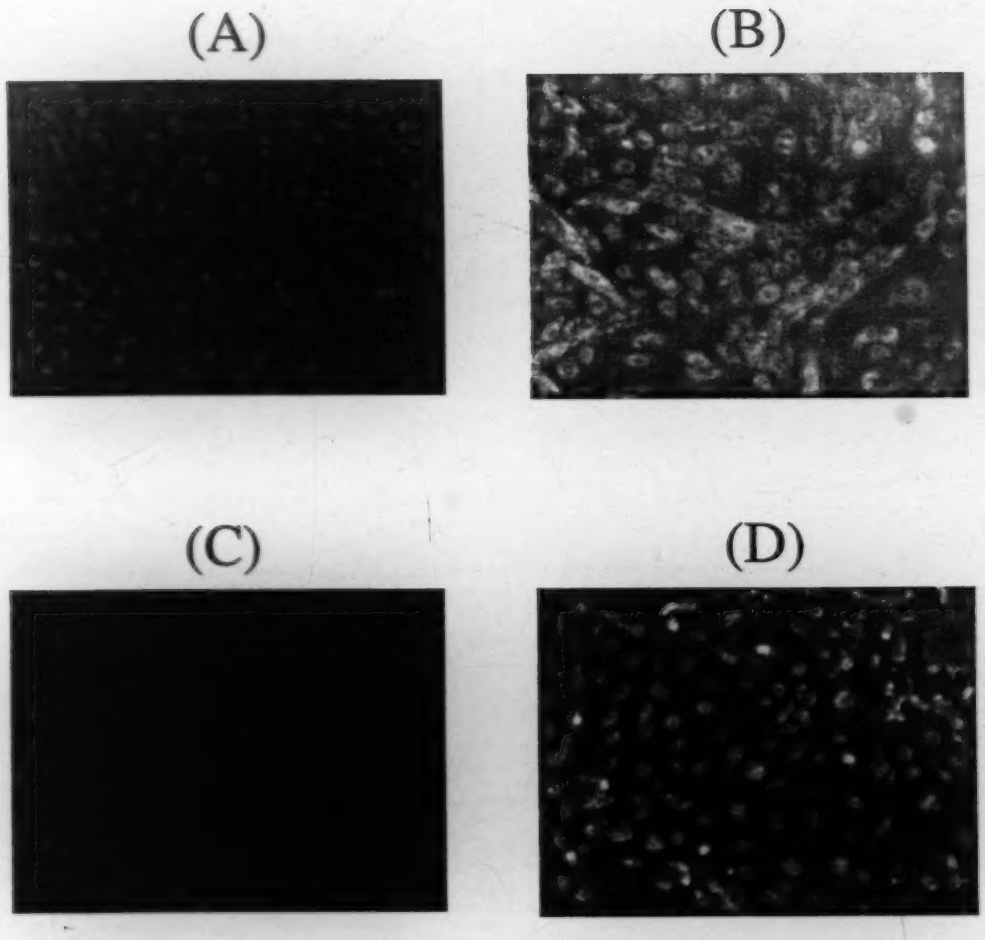

Figure A.1 Fluorescence immunostaining of HUVECs with endothelial-specific von Willebrand factor (vWF).

HUVECs were stained with DAPI (A) and vWF (B). Rabbit IgG controls are shown and were also stained with DAPI (C) and vWF (D).

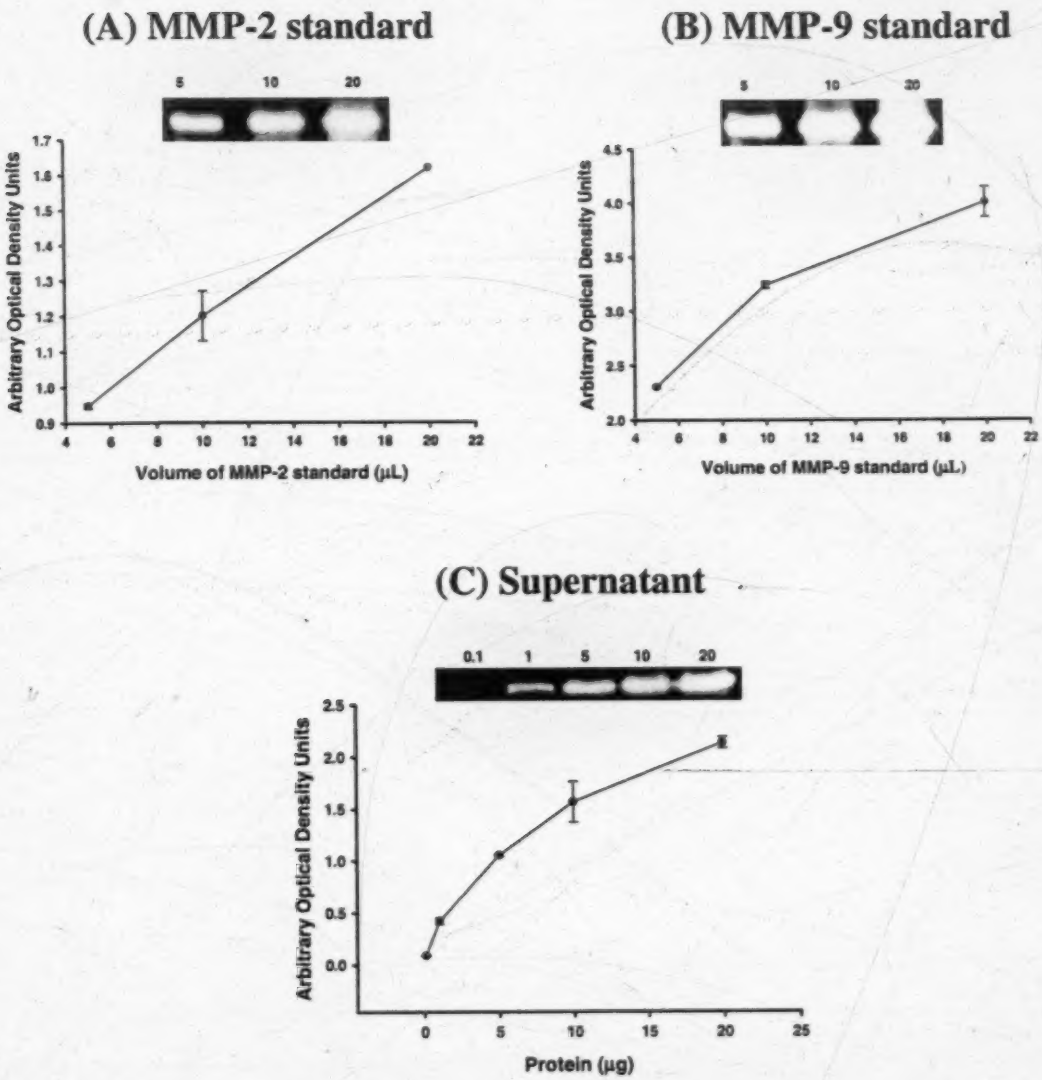

Figure A.2 Evaluation of the sensitivity of the zymography assay.

Increasing volumes (5, 10, 20 µL) of the MMP-2/9 standard were loaded into separate lanes of a gel (n = 2) and zymography was performed to construct dose-response curves for MMP-2 (**A**; 72 kDa) and MMP-9 (**B**; 92 kDa). A dose-response curve (n = 2) was also constructed using increasing amounts of protein from a supernatant sample (0.1, 1, 5, 10, 20 µg of protein; **C**; 72 kDa).

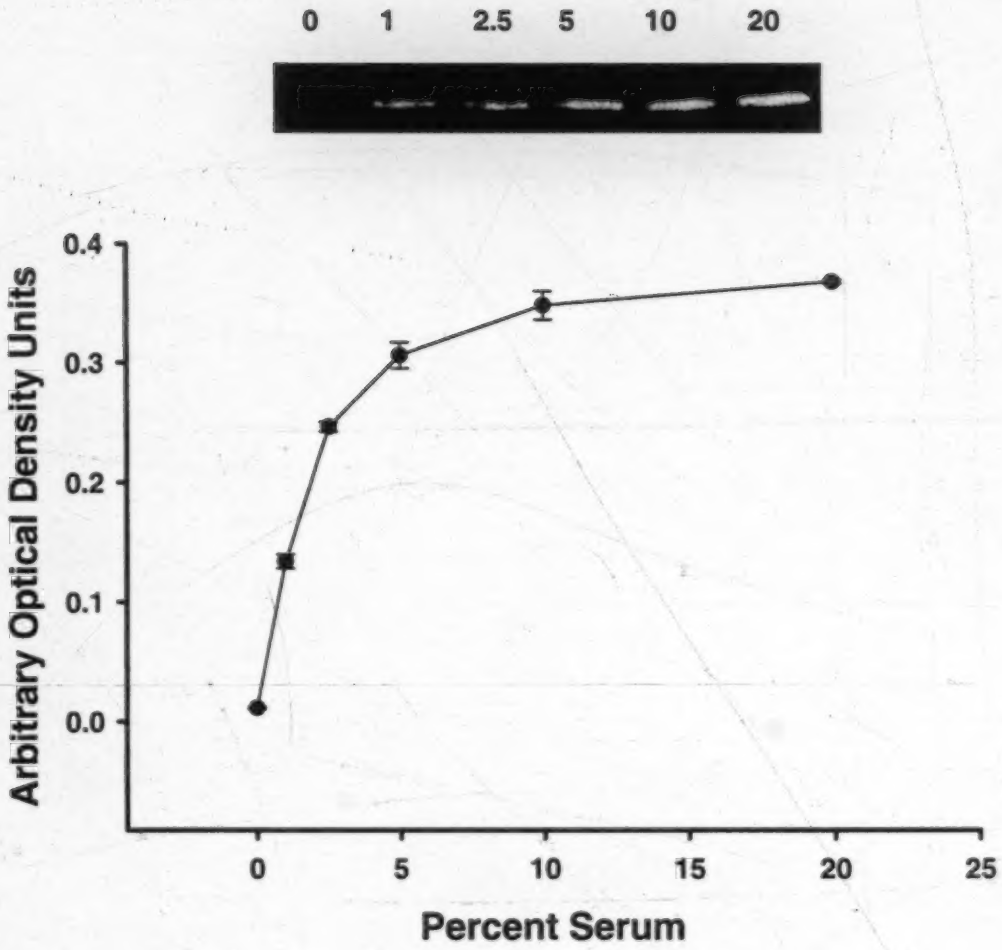

Figure A.3 Levels of endogenous metalloproteinases in serum-containing M199 medium.

Zymography was performed on samples of media containing increasing percentages of fetal bovine serum (0, 1, 2.5, 5, 10, 20%) in order to detect background levels of MMP-2 and MMP-9. MMP-2 (not MMP-9) was detectable in all serum-containing samples.

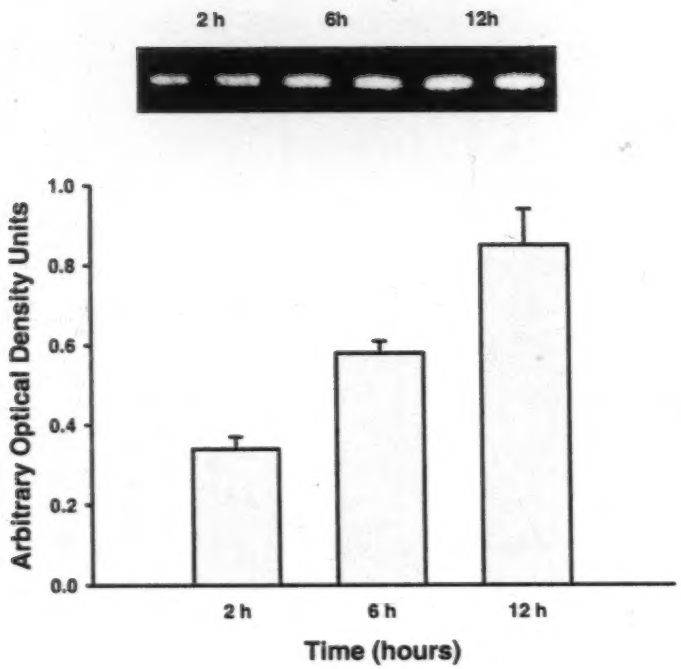

Figure A.4 MMP-2 accumulation in medium over time.

HUVECs from normal pregnancies were incubated under standard culture conditions for 2, 6, and 12 hours, and zymography was conducted on supernatants. The graph illustrates time-dependent MMP-2 (72 kDa) accumulation in medium.

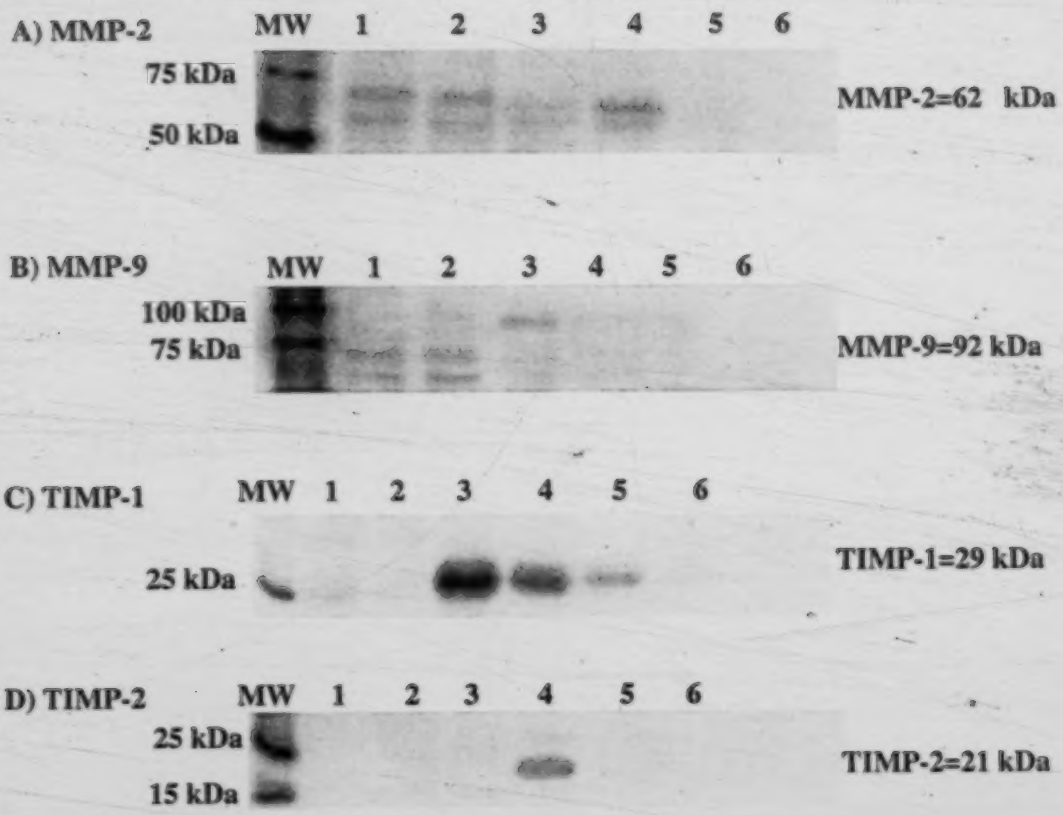

Figure A.5 Western blot analysis of conditioned medium from HT1080 cells as positive controls for MMP-2/9 and TIMP-1/2.

Cells were incubated overnight in the presence or absence of 100nm PMA and conditioned medium was concentrated using protein concentrators. The active (62 kDa) form of MMP-2 was detected in concentrated supernatant from unstimulated cells (**A**; lane 4). MMP-9 (92 kDa) was detected in conditioned concentrated medium from cells that had been stimulated with PMA (**B**; lane 3). TIMP-1 (29 kDa) was detected in all supernatant samples but was the most abundant in conditioned concentrated medium from cells that had been stimulated with PMA (**C**; lanes 3, 4, 5, and 6). TIMP-2 (21 kDa) was detected in the conditioned concentrated medium from unstimulated cells (**D**; lane 4). MW = molecular weight standard; 1 = stimulated cell lysate; 2 = unstimulated cell lysate; 3 = stimulated concentrated supernatant; 4 = unstimulated concentrated supernatant; 5 = stimulated unconcentrated supernatant; 6 = unstimulated unconcentrated supernatant.

HUMKDR 2530.F	GGC CAA GAG ATT GAA GCA GAT C
HUMKDR 3043.R	ACT TTC GCG ATG CCA AGA ACT C
HSFLT 2689.R	CCC ACT TGC TGG CAT CAT AAG G
HSFLT 2228.F	CAC CAT ACC TCC TGC GAA ACC T

Table B.1 **Sequence of oligonucleotide primers using for RT-PCR analysis of Flt-1 and KDR/Flk-1.**

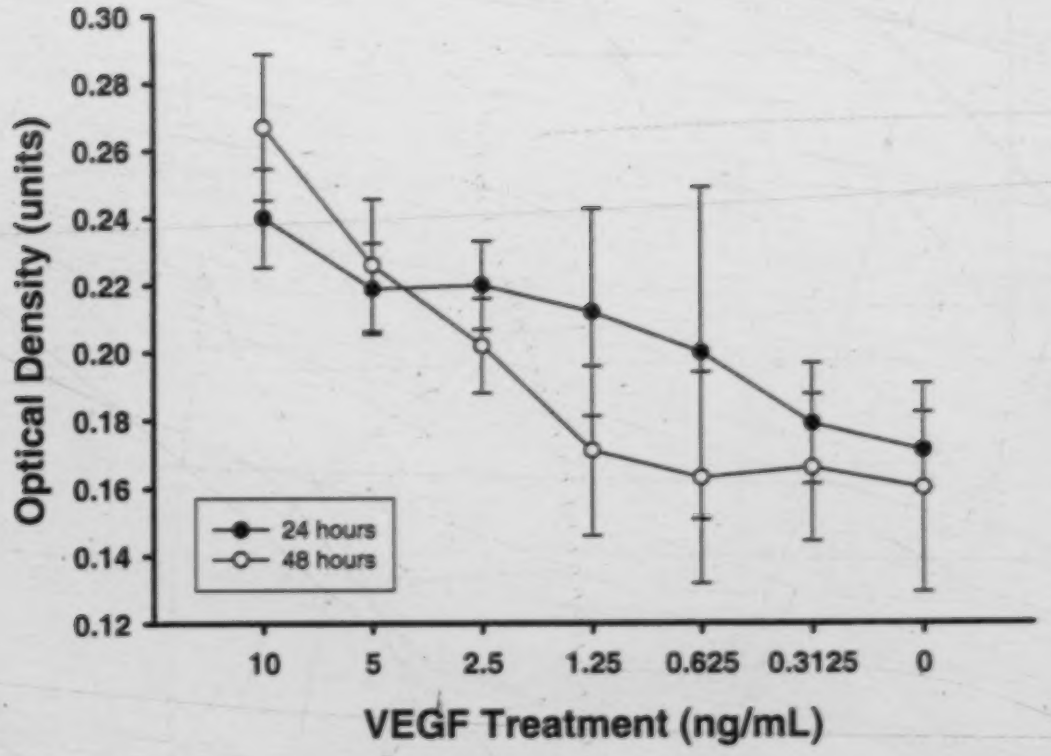

Figure B.1 The effect of VEGF on metabolic capacity in HUVECs from normal pregnancies.

HUVECs were incubated with doubling dilutions (0.31 - 10 ng/mL) of VEGF in medium containing 1% fetal bovine serum (n = 3). A dose-dependent effect of VEGF was observed at both 24 and 48 hours, with maximum metabolic capacity at 10 ng/mL VEGF.

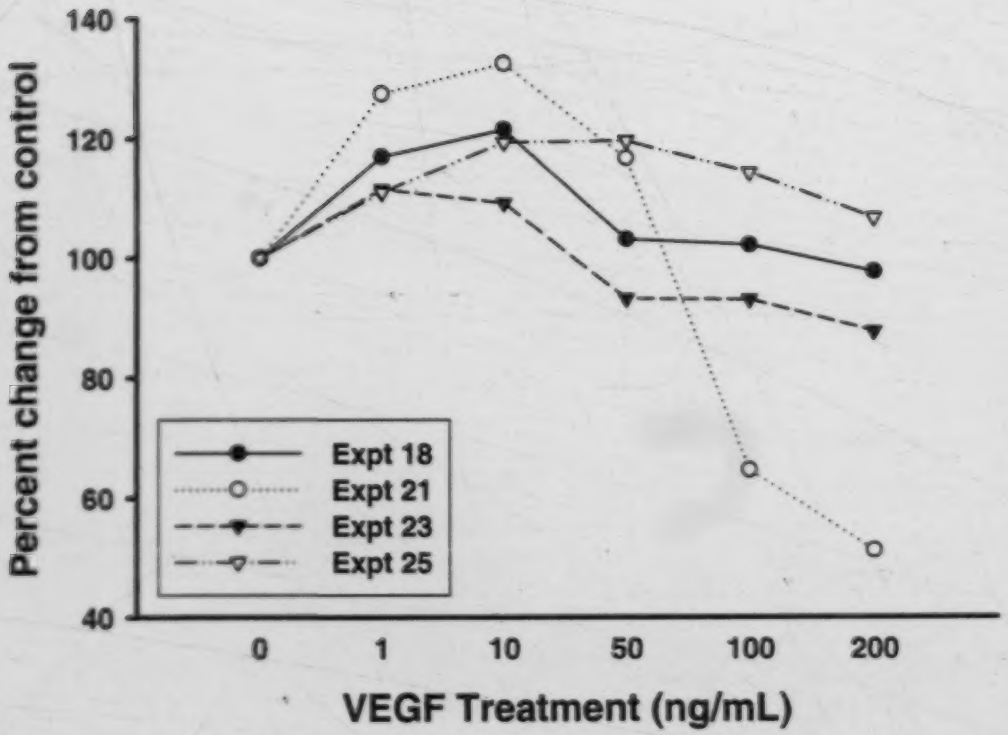

Figure B.2 The effect of VEGF on MMP-2 release from HUVECs.

Increasing concentrations of VEGF (1, 10, 50, 100, 200 ng/mL) were used to stimulate MMP-2 release from HUVECs (n = 4). VEGF stimulation did not significantly affect MMP-2 release in HUVECs at all concentrations tested (Friedman Repeated Measures ANOVA on Ranks; $p > 0.05$).

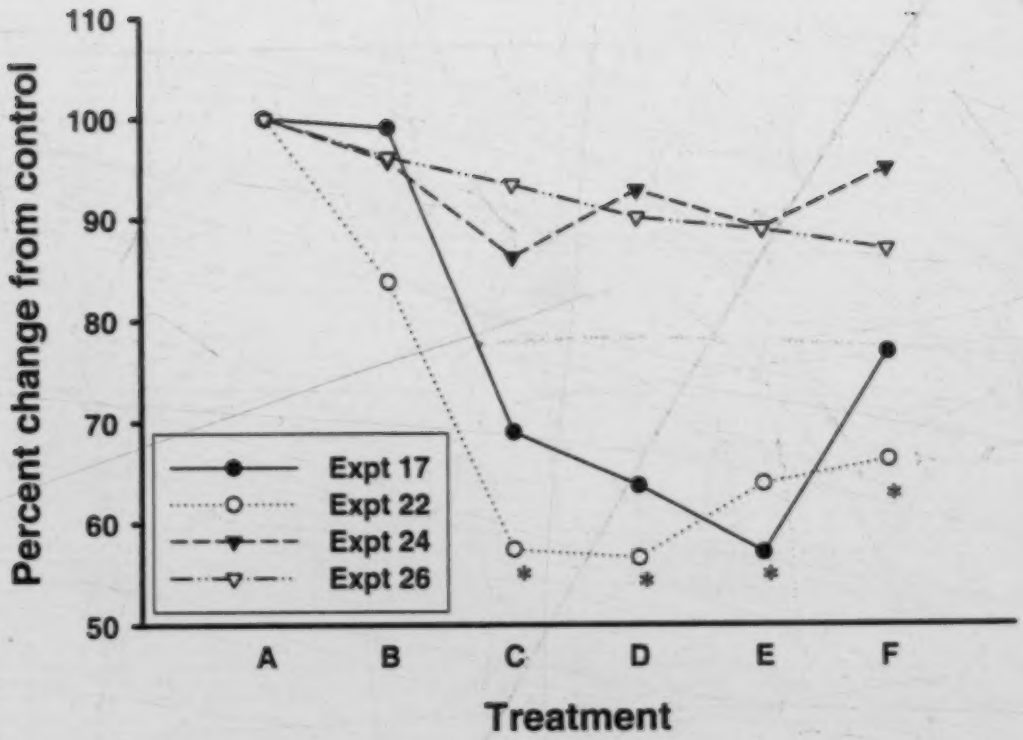

Figure B.3 The effect of endogenous and exogenous VEGF inhibition on MMP-2 release from HUVECs.

Two different concentrations of anti-human VEGF neutralizing antibody (0.3, 1.0 μg/mL) were tested in the presence and absence of exogenous VEGF (5.0 ng/mL; n = 4). The neutralizing antibody was found to significantly decrease MMP-2 release in the absence (*$p < 0.05$) and presence of exogenous VEGF (*$p < 0.05$) at both concentrations (Friedman Repeated Measures ANOVA on Ranks). A = control; B = 5ng/mL VEGF; C = 0.3 μg/mL anti-VEGF; D = 1.0 μg/mL anti-VEGF; E = 5 ng/mL VEGF + 0.3 μg/mL anti-VEGF; F = 5 ng/mL VEGF + 1.0 μg/mL anti-VEGF.

Figure B.4 RT-PCR analysis of Flt-1 and KDR/Flk-1 in HUVECs.

We confirmed the presence of the VEGF receptors Flt-1 (461 bp) and KDR/Flk-1 (513 bp) on HUVECs from normal pregnancies. Ladder = 100 base pair (bp) DNA ladder.